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VOL. 47 FEBRUARY, 1956 NO. 2

CLINICAL AND EXPERIMENTAL

A STUDY OF STIMULI LEADING TO THE PRODUCTION OF SPHERULES IN COCCIDIOIDOMYCOSIS

ORLAND BAKER, M.D., AND A. I. BRAUDE, M.D.
DALLAS, TEX.

INTRODUCTION

AN EXPLANATION for the formation of spherules in *Coccidioides immitis* has remained an enigma. The present investigation was undertaken to study their formation in an effort to explain the pathogenesis of coccidioidomycosis.

Coccidioides immitis is one of the pathogenic fungi which exhibits two stages in its growth cycle. In the saprophytic phase, it is a branching filamentous mycelium which reproduces by means of chlamydospores. In the parasitic phase, characteristically occurring only in infected tissues, it is a spherical structure which reproduces by endosporulation. Man probably acquires the infection when chlamydospores enter his lungs and are transformed into spherules. Once the fungus has established a focus of infection, it grows and reproduces only by spherule formation and endosporulation.[1]

Since spherules are the only form characteristically observed in vivo, the ability of *C. immitis* to produce infection and its consequent damage depends on the development of spherules from mycelia. The problem of spherule production was approached by first studying those factors which halt mycelial growth and next those which determine whether or not spherules emerge from the stunted mycelia.

From the Department of Internal Medicine, The University of Texas, Southwestern Medical School, Dallas, Tex.
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REVIEW OF THE LITERATURE

The parasitic phase of other pathogenic fungi having dimorphic growth cycles (*Sporotrichum schenckii, Blastomyces dermatitidis, Histoplasma capsulatum,* and *Candida albicans*) can easily be cultured in vitro by incubation at 37° C. on simple laboratory media containing such enrichment factors as blood or beef.[1] Attempts at in vitro cultivation of the parasitic phase of *C. immitis,* however, have produced inconstant results. Several workers have observed the production of spherules in media containing a variety of nutrients. MacNeal and Taylor[2] have reported spherule production under decreased oxygen tension in a medium of horse serum and slices of kidney tissue. Lack[3] observed spherule formation in glucose broth and in coagulated egg albumin under semianaerobic conditions. Baker and Mrak,[4] who reported the occurrence of "culture spherules" in several common solid media, observed that drying seemed to be an important factor in spherule production. They suggested that any condition which suppresses mycelial development would lead to the formation of spherules.

EXPERIMENTAL METHODS AND MATERIALS

A. *Source of Strains; Isolation; Identification.*—Strains of *C. immitis* studied in these experiments were isolated by inoculating Sabouraud's agar, Dubos medium, or mice with sputum, pus, or cerebrospinal fluid from five cases of the disseminated disease. All were patients in hospitals in Dallas, Texas, or vicinity. The oldest strain was isolated in October, 1953, from cerebrospinal fluid.

C. immitis was identified by finding spherules containing endospores in infected material from patients or by recovering such organisms through animal inoculation.

B. *Culture Media.*—Because of the hazard of laboratory infection, when arthrospores develop in aerial hyphae on agar, an effort was made to find a suitable liquid medium for *C. immitis.* Although the fungus grew readily in all common liquid media, Dubos medium (Difco) was chosen for three reasons: first, the wetting agent, Tween 80, by permitting dispersed mycelial growth, facilitated the preparation of uniform mycelial suspensions for inoculation of animals and preparation of mycelia-leukocyte suspensions; second, the formation of a pellicle with aerial mycelia was avoided because the mycelia grew near the bottom of the medium; third, arthrospores formed as rapidly in liquid media as on agar, so that the infectivity for animals was just as high. It was found that Tween 80 was not toxic for *C. immitis* and therefore albumin was not added to the Dubos medium. Stock cultures were maintained and transferred in 30 ml. rubber-stoppered vaccine vials containing Dubos medium.

C. *Technique for Making Lapse-Time Photomicrographs.*—Lapse-time photography is a means of greatly speeding up slight movements which are imperceptible to the eye. Very slow movement is greatly accelerated, so that what takes place in a matter of hours may be visualized in seconds. Individual pictures, or "frames," of movie film are exposed at intervals and later projected at a constant rate of 16 frames per second. In this way when the interval between frames is one hour, the events transpiring in 16 hours, for example, are visualized in one second.

Because this seemed a good method for studying the slowly growing hyphae, it was adapted to photomicrography to observe the behavior of normal mycelial growth. Ordinary slide cultures were not suited to the experiment because of the relatively great depth of field resulting from aerial hyphae. This would not allow perfect focusing on all hyphae at the same time. A more shallow depth of field was provided by the growth of arthrospores along the thin layer of Dubos medium, which became pulled up on the inner wall of the culture tube by capillary attraction. In this thin layer of nutrient material aerial hyphae did not form. A single plane of growth was thus formed which was ideally suited to photomicrography.

A 16 mm. Cine Kodak Special moving picture camera with a timing attachment for lapse-time work was used. The film was Eastman Super XX. The microscope, a Leitz mononuclear, was tilted so that the barrel was horizontal; this allowed for perpendicular

attachment of the culture tube to the stage so that the media would not spill. A Bausch and Lomb ribbon filament lamp supplied a source of light. The culture tube was rigidly attached to the stage of the microscope when a suitable field for study was located. The timing device was then set to take single frames at twenty-minute intervals. Enlargements were made at intervals from various frames for detailed study of the hyphal growth.

D. *Studies on the Hindrance of Mycelial Growth by Glass Beads.*—A sufficient number of spherical glass beads measuring 250 ± 25 μ in diameter were first placed in a test tube to a depth of 3 cm. To the tube was then added just enough Dubos medium to cover the beads. After sterilization, the tube was inoculated with coccidioidal mycelia. In order for spores to be distributed throughout the column of beads, a spinal needle measuring 4 inches in length was introduced to the bottom of the tube. As the needle was slowly withdrawn, mycelia were inoculated by means of a syringe. The tube was then stoppered and incubated at room temperature. When growth first appeared on top of the beads, the tube was opened and various depths along the column of beads were examined microscopically for growth. In addition, a flask of Dubos medium was inoculated with the deepest contents of the tube.

E. *Studies on the Hindrance of Mycelial Growth by Polymorphonuclear Leukocytes.*—

1. *Source of leukocytes and nonimmune plasma:* Twenty milliliters of blood was aseptically obtained by venipuncture from a nonimmune donor into a syringe moistened with a solution of saline containing heparin in a concentration of 10 mg. per milliliter. The blood was placed in a clean sterile tube and centrifuged at 500 r.p.m. for 5 minutes and then at 750 r.p.m. for 10 minutes. The plasma and buffy coat were next removed and placed in separate tubes. The plasma was centrifuged at 2,000 r.p.m. for 10 minutes to obtain cell-free plasma. The buffy coat was next washed twice with Ringer's lactate and centrifuged at 1,000 r.p.m. for 5 minutes after each washing. The cells were finally resuspended in 0.5 ml. Ringer's lactate.

2. *Killed leukocytes:* Studies with living leukocytes were repeated with dead leukocytes. Destruction of the cells was accomplished by suspending them in Ringer's lactate and heating at 60° C. for 10 minutes.

3. *Immune plasma*: Plasma was obtained from the five patients with disseminated infection whose strains of *C. immitis* were being studied and was stored at -25° C. until ready for use. The complement-fixation titers* of these plasmas ranged from 1:32 to 1:4,096.

4. *Mycelial suspensions*: Vials containing mycelial culture in Dubos medium were agitated in a paint shaker to disperse the endospores and break up the mycelia. Examination revealed mycelial segments containing from 2 to 50 chlamydospores. The contents of the vial were then centrifuged at 1,500 r.p.m. for 5 minutes and the sediment washed three times with Ringer's lactate. The mycelial fragments were then resuspended in 0.5 ml. of Ringer's lactate. All five strains of mycelia were treated in this fashion.

5. *Indirect observations of neutrophil activity with stained preparations:* Aliquots of the mycelial suspension of a given strain were placed in seven vials. To the first vial of each strain was added 5 ml. Dubos medium. To the second vial was added at six-hour intervals freshly prepared suspensions of washed living leukocytes. The third vial received killed leukocytes from a freshly prepared suspension of washed white cells. To the fourth vial was added fresh nonimmune plasma; to the fifth, immune plasma; to the sixth, nonimmune plasma plus freshly washed leukocytes; and to the seventh, immune plasma plus freshly washed leukocytes. Every six hours, a fresh supply of all constituents was added to the mycelia. The nonimmune plasma and leukocytes were obtained from the same donor just before each replenishment. The vials were incubated at 37° C. and watched for growth.

A hypodermic needle was used to withdraw 1 drop of material from each vial just before the addition of fresh cells or plasma. The drop was then placed on a clean slide and spread with the tip of the needle. When the slides dried they were fixed with absolute methanol for 24 hours to assure killing of the fungus. The slides were then stained with buffered Wright's stain and observations made for microscopic appearance of the fungus.

6. *Direct observations of neutrophil activity:* Direct observations of mycelia-leukocyte suspensions were made by means of wet preparations. Blood was obtained from a nonimmune

*The complement-fixation tests were performed either by Dr. C. E. Smith of the University of California or at the Communicable Disease Center in Chamblee, Georgia.

normal person for control and from a patient with coccidioidomycosis whose strain of *C. immitis* was used for the study.

Twenty milliliters of blood was aseptically obtained by venipuncture from both the normal person and the patient and placed in separate sterile tubes containing 2 mg. heparin. The tubes were centrifuged at 500 r.p.m. for 5 minutes and then at 750 r.p.m. for 10 minutes, after which the plasma and buffy coat were removed and placed in separate tubes. The two samples of buffy coat were next washed twice with Ringer's lactate and centrifuged at 1,000 r.p.m. for 5 minutes between washings. The leukocytes were finally suspended in 0.5 ml. of Ringer's lactate. Volumes of 0.25 ml. of the leukocyte suspension were combined with equal volumes of plasma as follows: normal leukocytes with normal plasma; normal leukocytes with patient's plasma; patient's leukocytes with normal plasma; and patient's leukocytes with patient's plasma. The cell suspensions were gently shaken by hand and one drop of each of the four suspensions placed on separate clean glass slides.

A mycelial culture in Dubos medium was shaken to fragment and disperse the mycelia. One drop of this culture was then placed on each of the four slides near the drop of suspended leukocytes. The two drops were then mixed with the tip of a pipette and a cover slip was applied and sealed to prevent evaporation. The slides were studied microscopically for phagocytosis.

F. *Anaerobic Methods.*—Six cotton-stoppered tubes of Dubos medium were inoculated with mycelial suspensions. One tube was allowed to remain at atmospheric conditions and room temperature. The other five tubes were sealed inside five Brewer anaerobic jars. Each jar was evacuated to 4 mm. Hg pressure and the vacuum replaced by illuminating gas. This procedure of evacuation and gas replacement was repeated six times in order to flush out of the jars all but a trace of the original air. Finally, the jars were evacuated and oxygen was added in concentrations of 10 per cent, 5 per cent, 2.5 per cent, and 1.25 per cent to each of the first four jars. The fifth jar received no added oxygen. The remaining vacuums were filled with illuminating gas.

When growth appeared in the control tube at room temperature, the tubes were removed from the Brewer jars. Each tube was shaken and half its contents removed and centrifuged. The sediment was examined microscopically. The other halves of the contents of the tubes were left in atmospheric environment and observed a few days later for growth. This entire procedure was carried out on each of the five strains of *C. immitis.*

G. *Inoculation of Irradiated Mice.*—Fifty male albino mice, weighing 20 to 25 grams, were given 450 roentgens of total body irradiation. This was done at 220 kv. 18 Ma. with an x-ray therapy unit which delivered 60 roentgens per minute. The irradiation was given at a distance of 20 cm. through 0.5 cm. copper and 1 cm. aluminum filters. The mice were placed inside a round glass jar approximately 25 cm. in diameter and 4 cm. deep. The jar was covered with a thin plastic membrane to keep the mice from escaping during irradiation. Ten mice were irradiated at one time. Daily white cell counts were done on tail blood. A maximum degree of leukopenia occurred on the fifth day.

1. *Source of mycelia for inoculation:* A mycelial culture in Dubos medium which had undergone no more than six transfers since original isolation was agitated in a paint shaker for 15 minutes. Direct microscopic count revealed approximately 5,000 hyphal fragments per milliliter. This was diluted 1 to 10 with normal saline, and 0.5 ml. (or a total of approximately 250 hyphal fragments) was used for each inoculation.

2. *Source of spherules for inoculation:* Pus was aspirated from a subcutaneous abscess on the patient's forearm. Fifty μg streptomycin and 100 units of penicillin per milliliter were added to the pus. Microscopic count revealed approximately 1,400 spherules per milliliter. Two-tenths milliliter of pus (a total of approximately 280 spherules) was used for each inoculation.

3. *Inoculation*: When the white cell counts of the irradiated mice were at the lowest level, half were inoculated intraperitoneally with pus (spherules) and half with the mycelial suspension. Fifty controls were similarly inoculated.

4. *Killing mice:* At intervals of 2 hr., 4 hr., 8 hr., 12 hr., 24 hr., 48 hr., 3 days, 4 days, 5 days, 6 days, and 8 days after inoculation, two mice in each group were sacrificed by etherization. The remaining mice were allowed to die of coccidioidal infection. The peritoneal cavity was opened and examined for evidence of infection. The method used

for grading the degree of infection is illustrated in Fig. 1. Grade 1 signified lesions confined to the site of inoculation, greater omentum, or localized area of peritoneum. Grade 2 infection had more extensive lesions involving wider areas of the peritoneum, the epididymis, spleen, and liver. Grade 3 infection included lesions on the dome of the liver and the diaphragm as well as more numerous lesions in the spleen, mesentery, peritoneum, epididymis, and liver. Grade 4 infection signified involvement of the lungs in addition to that of grade 3.

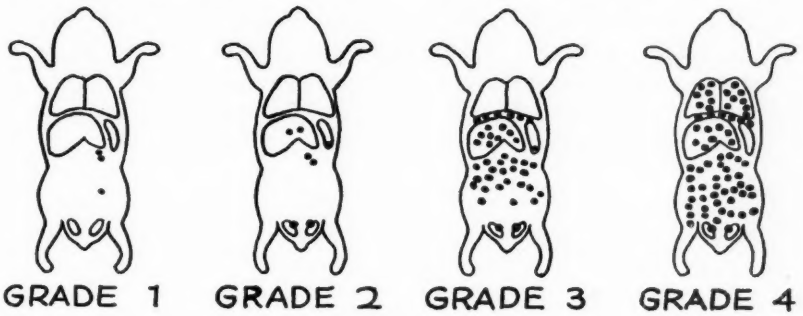

Fig. 1.—Grading of degree of infection in mice with *Coccidioides immitis*.

EXPERIMENTAL RESULTS

The process by which mycelia are converted into spherules was investigated first by studying those factors which halt mycelial growth and next those factors which determine whether or not spherules emerge from the stunted mycelium.

A. *Spatial Hindrance by Obstructing Hyphae.*—The first lead was obtained from observations made on normal growth of coccidioidal mycelia by means of lapse-time photomicrographs of the fungus. These showed that hyphae always stopped growing when obstructed by other hyphae and never crossed over each other. In Fig. 2 the results of this experiment are illustrated by six frames chosen at crucial intervals from the lapse-time film. Obstructing hyphae (shown by arrows) stopped the growth of advancing hyphal tips. Occasionally, an advancing hyphal tip was able to change direction of growth and thus avoid the obstruction (see arrow A, Fig. 2). Although spatial hindrance halted mycelial growth, no spherules developed.

B. *Spatial Hindrance by Glass Beads.*—Spatial hindrance was also produced by placing glass beads in the culture medium. When liquid media was inoculated with *C. immitis,* mycelial growth occurred in the bottom of the tube. However, when closely packed minute glass beads were placed in the culture medium, no mycelial growth occurred in the bottom of the tube. Growth occurred only above the beads. When the culture tube was broken and the depths of the tube examined, no growth was apparent either grossly or microscopically. Yet, subsequent inoculation of a flask of media with this material yielded growth. In other words, growth was prevented by the mechanical obstruction, but the mycelia were not killed.

C. *Spatial Hindrance by Polymorphonuclear Leukocytes and the Emergence of Spherules.*—It was considered likely that spatial hindrance might occur in vivo if neutrophils attacked the mycelium. This idea was borne out by the following series of investigations:

1. ***Leukocyte suspensions in vitro:*** Mycelia were found to be vigorously attacked and phagocytosed by neutrophils when direct observations were made of

living leukocyte suspensions. Immediately after being mixed with mycelia, neutrophils migrated directly toward them. Upon reaching the mycelium, the neutrophil immediately adhered and the cytoplasm flowed around the mycelial fragment. Single arthrospores or very short mycelial segments were completely engulfed (Fig. 3). Longer segments were encircled by the cytoplasm of the neutrophil, which formed a wide band around the mycelium. This neutrophilic activity proved futile, however, because dissolution of the mycelial wall never occurred. After 4 to 6 hours, the phagocytic cells began to show evidence of decreasing activity; fewer pseudopods were seen and the cells began

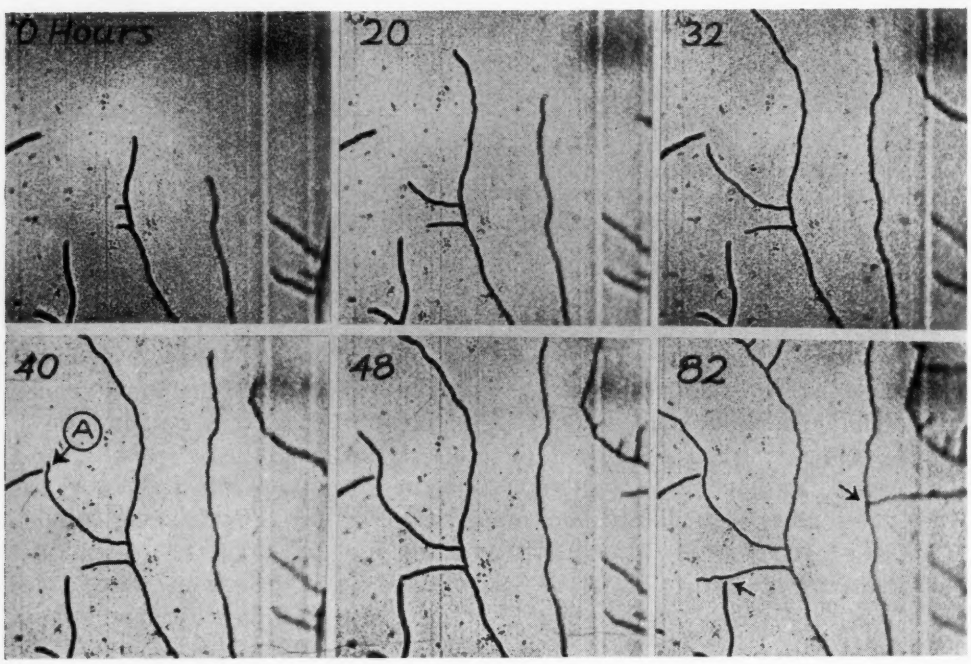

Fig. 2.—Lapse-time photomicrographs showing mycelial hindrance by obstructing hyphae (at arrows). Growth on inside wall of test tube (×200; reduced ⅕).

to swell and disintegrate. The speed and outcome of this process were identical regardless of whether leukocytes or plasma were obtained from patients with coccidioidomycosis or from normal individuals.

When mycelia were added to normal human plasma containing a supply of frequently replenished living human neutrophils, mycelial growth was invariably prevented; yet mycelia grew freely in plasma containing dead neutrophils or no neutrophils. In such suspensions of living leukocytes in plasma, it was not only possible to demonstrate vigorous phagocytosis but also spherule formation. With all five strains studied, it was possible to follow the stages of spherule formation. Fig. 4 consists of photomicrographs of a series of stained smears made at intervals during the incubation of mycelia with leukocytes. The earliest smear made two hours after the beginning of the experiment shows normal mycelium with segmented arthrospores being attacked by neutrophils.

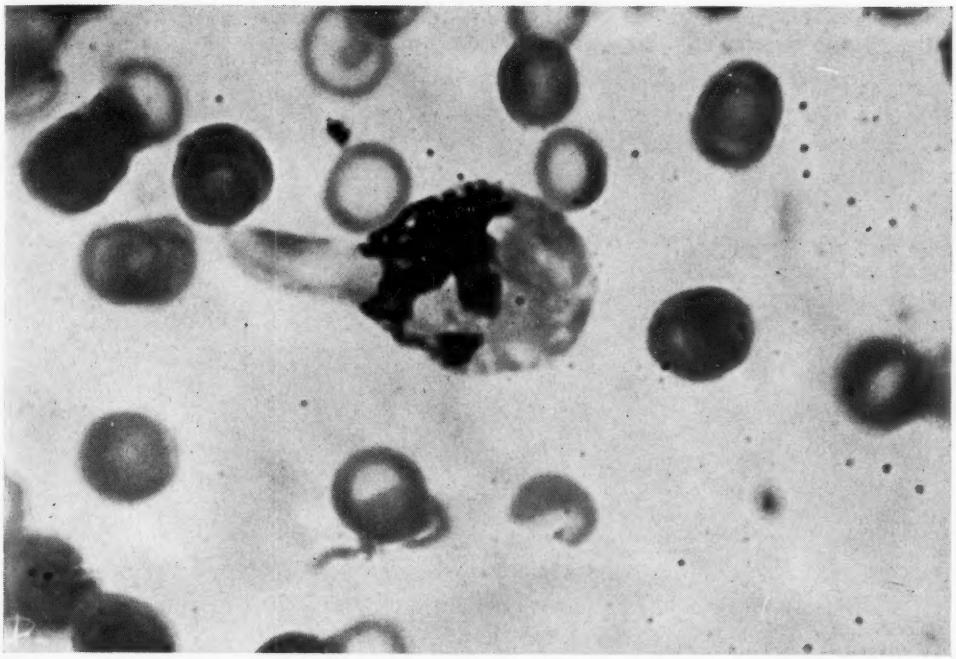

Fig. 3.—Neutrophils phagocytosing short mycelial fragment 10 minutes after addition of mycelia to leukocyte suspension from normal individual. Neutrophil has engulfed all but a tip of the mycelium (Wright's stain, ×2,200; reduced ¼).

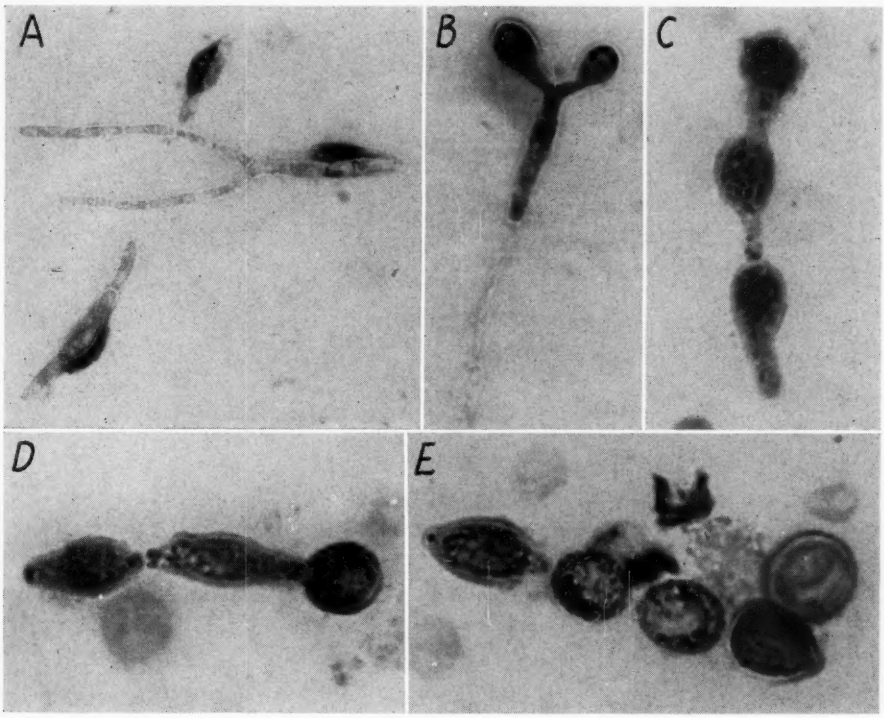

Fig. 4.—Formation of spherules in vitro. Fresh normal neutrophils in normal plasma were added to mycelia at 6-hour intervals. Slides were made at 2-, 12-, 48-, 78-, and 108-hour intervals showing progressive stages of spherule development from mycelia (Wright's stain, ×2,200; reduced ½.) *A*, 2 hr.; *B*, 12 hr.; *C*, 48 hr.; *D*, 78 hr.; *E*, 108 hr.

By 12 hours there was segmental swelling of some of the mycelia and beginning degeneration of other segments. At 48 hours definite segmentation had occurred and only pale-staining ghosts of the original mycelia were observed. At 78 hours the first doubly refractile thick-walled spherules containing endospores were seen. These continued to be more numerous in subsequent smears, and by 108 hours no mycelia were found. Definite spherules with endospores had developed. Mycelial inhibition and spherule formation occurred whether leukocytes were suspended in normal plasma or in plasma with high antibody titers from patients whose strains were being tested. When cells were suspended in

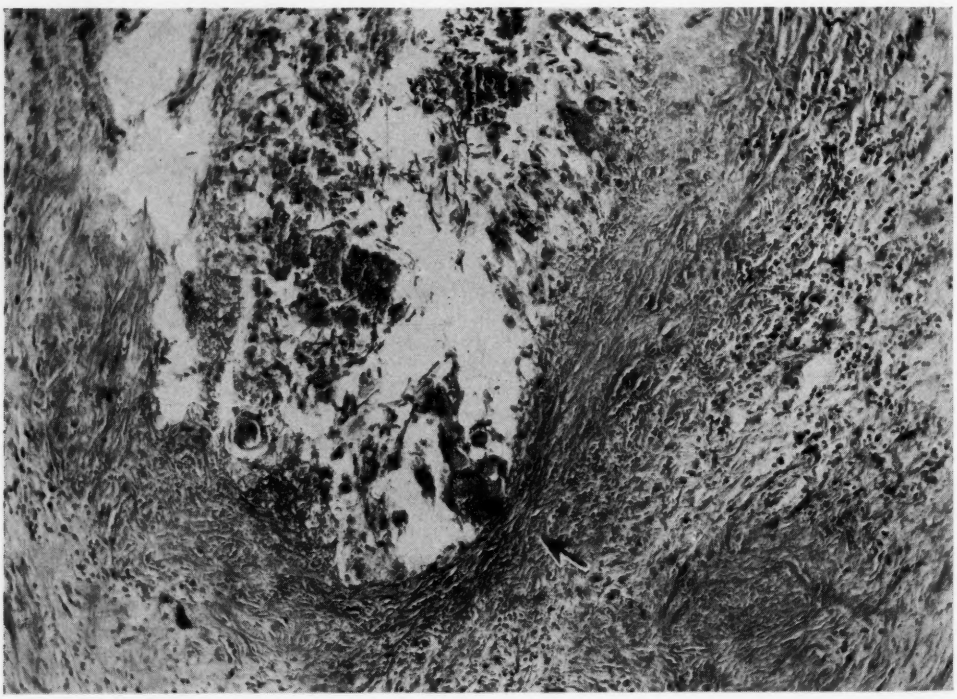

Fig. 5.—Meningeal focus of infection from a patient dying of coccidioidal meningitis. Base of brain was covered by a thick plastic exudate. Disease was 6 months old. Note: (1) thick fibrous capsule around lesion, (2) mycelia in center of lesion, (3) spherules at peripheral point of lesion (at arrow), (4) absence of neutrophils within lesion. (Hematoxylin and eosin, ×140; reduced ⅕.)

the plasma with high titers of antibody, however, spherule formation seemed to occur at a slightly faster rate than when normal plasma was used.

2. *Leukocytic activity in infected tissue:* It was also possible to provide in vivo evidence that the presence of neutrophils influences spherule formation.

Nonirradiated tissue: In a search through human tissues, mycelia were found only in walled-off pulmonary cavities and within an acellular meningeal focus. In both of these locations, the striking absence of leukocytes suggested that mycelial development was inhibited by the presence of leuokcytes but that mycelia are free to develop in their absence. Examination of tissues in a fatal case of coccidioidal meningitis disclosed the lesion shown in Fig. 5. This lesion was surrounded by a thick fibrous capsule which prevented the entrance of

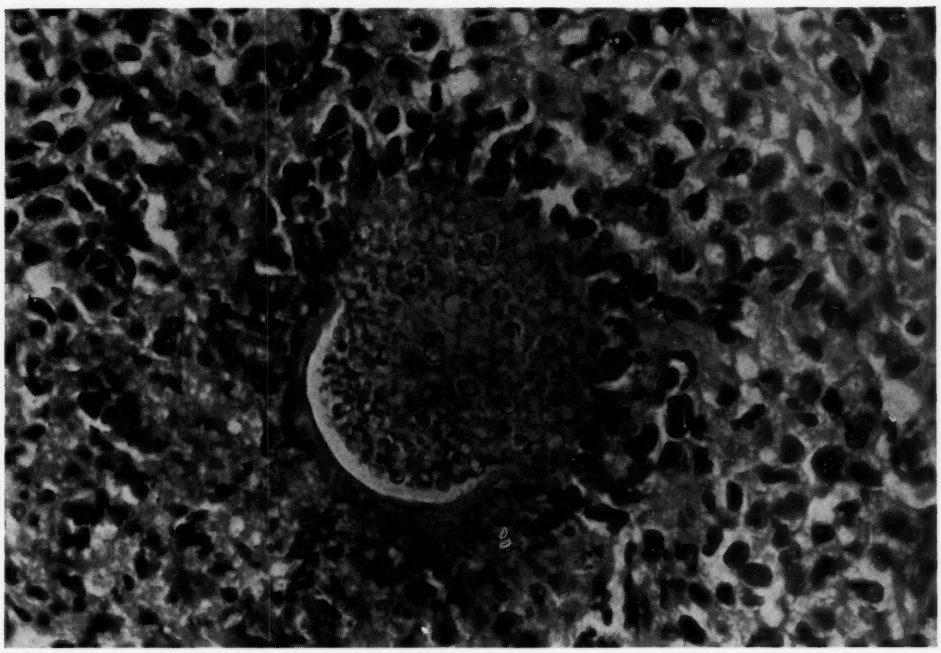

Fig. 6.—Neutrophils attacking expelled contents of a rupturing spherule. Section of mouse liver (hematoxylin and eosin, ×800; reduced ⅓).

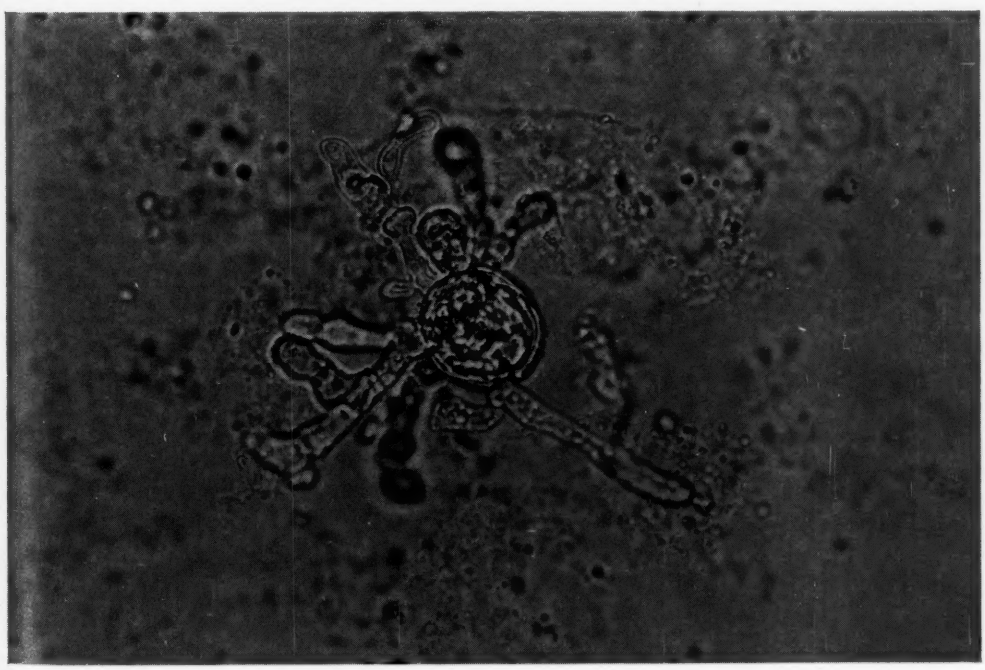

Fig. 7.—Mycelia emerging from spherule less than 6 hours after inoculating pus in Dubos medium (×900; reduced ⅓).

leukocytes into the center of the lesion, although a considerable number of cells were found outside the capsule. Mycelia can be observed within the capsule at the center of the lesion, while spherules are present only at the very periphery adjacent to an aggregation of leukocytes (see arrow, Fig. 5). There is also an absence of mycelia in the immediate vicinity of the leukocytes. The concentration of white cells in the vicinity of the spherules lends evidence that leukocytes influence spherule formation.

Additional evidence connecting neutrophil activity with spherule formation was provided by examination of infected animal tissues. In the presence

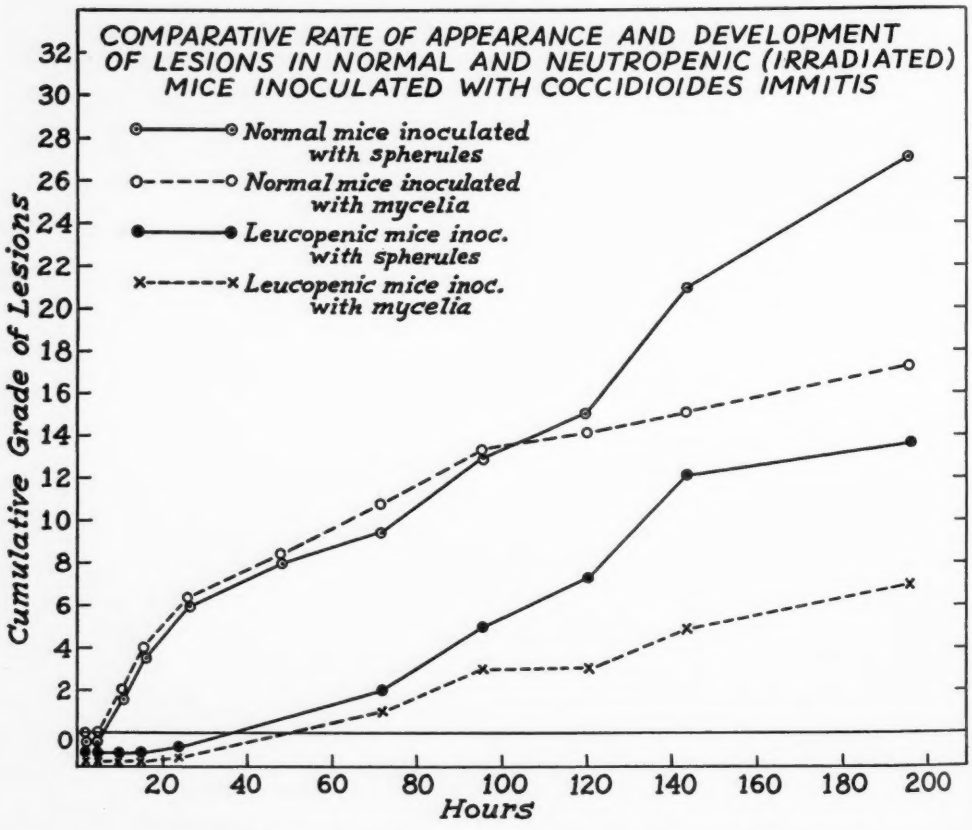

Fig. 8.—Relative rate at which lesions develop from mycelia and spherules in normal and leukopenic mice. Accumulation of lesions in consecutive mice is plotted on the basis of grading system in Fig. 1.

of the intact thick-walled spherule, few neutrophils were in evidence and the cellular reaction was chiefly epithelioid. When the spherule ruptured, however, neutrophils poured in to attack the expelled spores (Fig. 6). Neutrophils not only attacked and phagocytosed the endospores but also moved inside the spherule and attacked the shell lining. The endospores developed into mature spherules whose protecting wall again insulated the surrounding tissues from the leukotactic material contained within the spherule. If, on the other hand, the spherule was placed in culture medium, in the absence of neutrophils, the endospores gave rise directly to mycelia (Fig. 7).

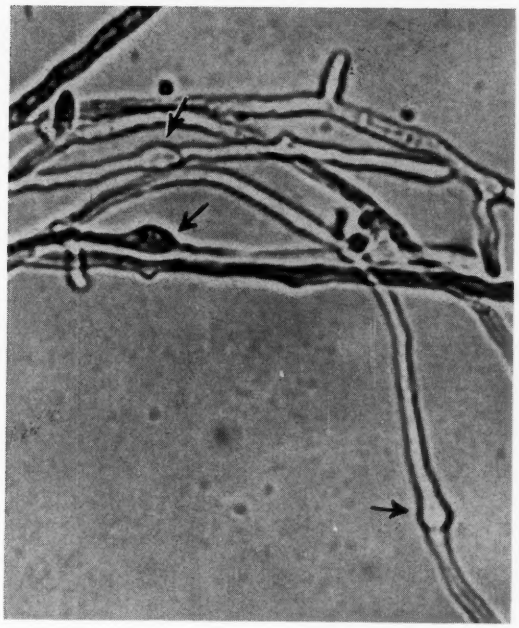

Fig. 9.—"Abortive spherules" (at arrows) seen in mycelia in anaerobic environment (Wright's stain, ×1,240; reduced ⅕).

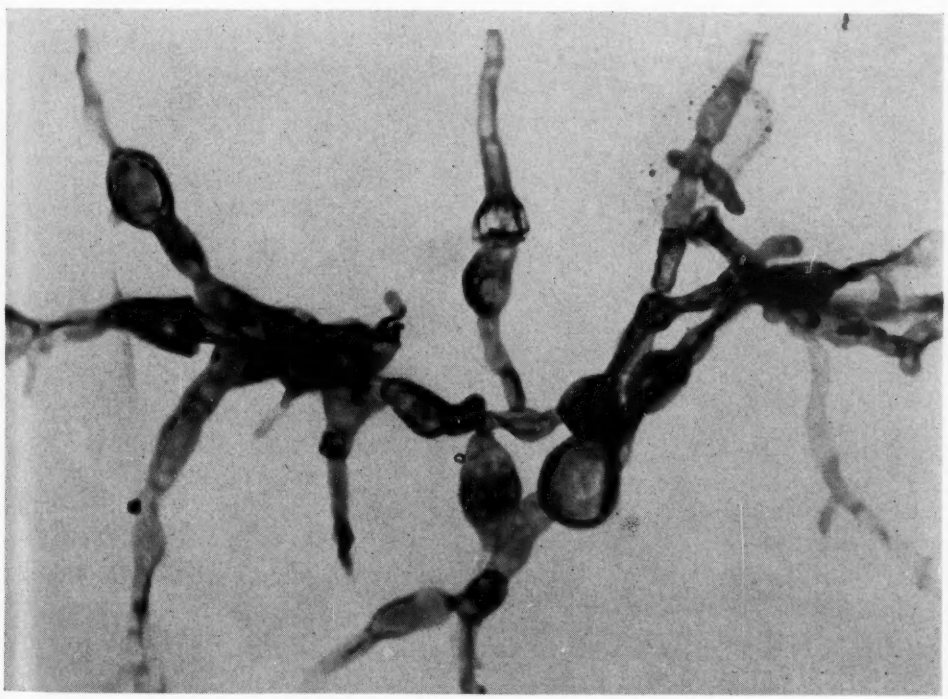

Fig. 10.—"Abortive spherules"—mycelial fragment recovered from peritoneal exudate two hours after intraperitoneal inoculation of a mycelial suspension in a mouse (Wright's stain, ×1,200; reduced ⅙).

Irradiated tissues: If neutrophils are responsible for the conversion of mycelium to spherules in vivo, it should be possible to retard the emergence of spherules by reducing the supply of neutrophils. When this hypothesis was tested, it was found that lesions developed more slowly in irradiated neutropenic mice inoculated with mycelia than in those inoculated with spherules. In nonirradiated controls, with abundant neutrophils, lesions developed from mycelia and from spherules at the same rate. Fig. 8 illustrates this finding graphically. Because development of lesions depends on the presence of spherules, a delay in spherule formation from mycelia thus occurred in neutropenic mice.*

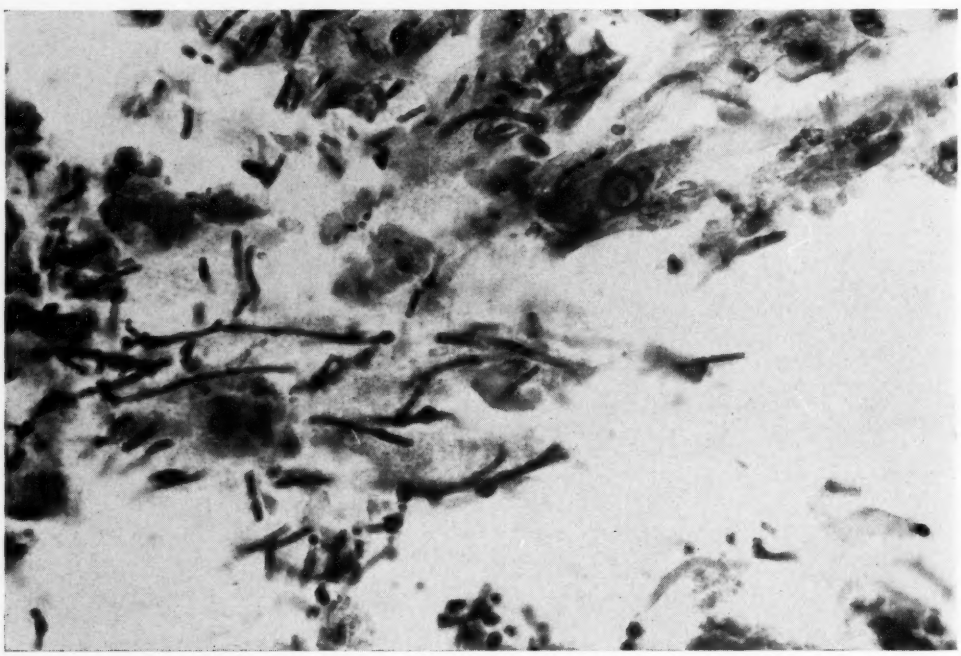

Fig. 11.—"Abortive spherules"—enlargement of central portion of meningeal focus in Fig. 5, showing swellings of mycelia. (Hematoxylin and eosin, ×650; reduced ¼.)

D. *Anaerobiosis:* Mycelial growth was also studied under conditions of variable oxygen tension because the avascularity of coccidioidal granulomas suggested that anoxia might be a factor in mycelial inhibition. It was found that mycelia grew readily in concentrations of oxygen greater than 2.5 per cent, but in conditions approaching complete anaerobiosis, there was no growth until the tube was restored to atmospheric conditions. In anaerobiosis, bodies resembling "abortive spherules" formed in the mycelia. These appeared as swellings and thickenings of the mycelial wall (Fig. 9, see arrows). The appearance of these "abortive spherules" helps explain certain stages of mycelial development observed in the tissues. First, they resemble early stages of spherule formation in mycelia which have recently been inoculated intraperitoneally in

*The late increase in lesions (after the 120th hour) in both normal and irradiated mice inoculated with spherules over those inoculated with mycelia is explained by the greater number of potentially infective units contained within each spherule.

mice (Fig. 10). Also, they are identical in appearance to swellings seen on mycelia in the walled-off meningeal focus where oxygen tension is very low (Fig. 11). Although anaerobiosis prevents mycelial growth, it also prevents full development of normal spherules even in the presence of attacking neutrophils.

DISCUSSION

There are two distinct phases in the development of spherules from mycelia: first is the inhibition of mycelia, and second, the emergence of spherules from the stunted mycelia. Mycelial inhibition results from the spatial hindrance provided not only by such stationary objects as obstructing hyphae or glass beads but also by attacking neutrophils. Since spherule formation occurs in vitro only when living leukocytes are added to mycelial cultures, it appears that the formation of spherules is a mechanical phenomenon which depends on the type of spatial hindrance provided by living motile neutrophils ready to counter every advancing hyphal tip and completely prevent mycelial development. In the absence of neutrophils in vitro, the organism develops in the natural mycelial form, but when attacked by leukocytes, it is forced into the more resistant spherule in order to multiply. From these studies it must be concluded that the neutrophils provide an inadequate cellular defense mechanism against the highly pathogenic strains of *C. immitis* used in this study. Although their attempt at phagocytosis is vigorous, they not only fail to destroy the fungus but they also encourage the formation of the destructive spherule. The observation that neutropenic animals experienced milder infections, with less tissue damage than normals, illustrates the futile and even harmful effect of the leukocytes in coccidioidomycosis. It is possible, however, that strains which produce mild or no clinical disease may be more vulnerable to the attack by neutrophils than those selected for study here. Their greater vulnerability might represent: (1) an inability of mycelia to convert to spherules; in this case, spatial hindrance by neutrophils would result in arrest of fungal growth; or (2) greater susceptibility of the arthrospores themselves to destruction by neutrophils.

SUMMARY

Two distinct stages were observed in the formation of spherules by virulent strains of *C. immitis*. First was inhibition of mycelial growth. Second was the emergence of spherules from the stunted mycelium. Although mycelial inhibition resulted during natural growth when hyphae obstructed each other and experimentally when immobile objects as glass beads were present in the medium, spherules did not appear. The emergence of spherules occurred only when mycelial inhibition was accomplished by the vigorous attack by living motile neutrophils.

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VIREMIA IN MICE WITH PULMONARY INFLUENZA A VIRUS INFECTIONS

DOROTHY HAMRE, PH.D., JASON APPEL, M.D., AND CLAYTON G. LOOSLI, M.D.
CHICAGO, ILL.

THE present study was carried out to obtain, if possible, quantitative data on the incidence and duration of viremia in mice infected intranasally or by aerosol with mouse-adapted influenza A (PR8) virus. Our interest in this problem arose during studies concerning the immunologic mechanisms in experimental influenza A infections in mice.[1] It was noted that mice passively immunized with large doses of high titer rabbit antisera and infected by allowing them to breathe highly lethal air-borne doses of influenza A (PR8) virus, developed infections but did not die. Although growth of virus in the lungs occurred, circulating antibodies could not be detected following recovery. This was interpreted as being due possibly to the presence initially of passive neutralizing antibody which immediately inactivated any virus which might have entered the bloodstream during its growth in the lung, and thus prevented it from provoking a generalized immune response.

MATERIALS AND METHODS

Mice.—Swiss albino mice, Webster strain, of both sexes, weighing 18 to 25 grams were used. They were shown by serial passage of lungs to be free of latent pulmonary viruses.

Virus.—The mouse-adapted PR8 strain of influenza virus A was employed. It was originally obtained from Dr. Thomas Francis, Jr. in 1941, and has been maintained in the laboratory by lung passage in mice at two- to three-month intervals. Stock passage virus was prepared by exposing mice in a closed chamber to highly lethal clouds of nebulized 10 per cent mouse-lung suspension of virus. After forty-eight hours the animals were sacrificed, their lungs removed aseptically, and ground in a mortar with alundum and antibiotic broth (sterile broth containing 1,000 units of penicillin and 1 mg. of dihydrostreptomycin per milliliter) to make a 10 per cent suspension by weight. After slow centrifugation for five minutes, the supernate was separated in aliquots of 5 ml., quickly frozen, and stored at –50° C. in an electric Deepfreeze. Such virus suspensions were found generally to have an EID_{50} titer of $10^{9.0}$ to $10^{9.5}$ and a mouse LD_{50} titer of $10^{7.0}$ to $10^{7.5}$.

Air-borne Technique of Infecting Mice.—In the majority of experiments mice were infected by allowing them to breathe nebulized mouse-lung virus suspension in the cloud chamber. The chamber had a capacity of 170 L. and was similar in design but larger than that employed in previous studies.[2, 3] The mice were introduced into the chamber in compartmented wire cages to prevent crowding. In a given experiment, a known dilution of lung virus suspension was placed in a DeVilbiss (No. 40) atomizer which was weighed and attached to the chamber. The material, usually 5.5 ml., was then nebulized into the

From the Section of Preventive Medicine, Department of Medicine, University of Chicago School of Medicine, Chicago, Ill.

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chamber with air under a pressure of 500 mm. Hg for twenty minutes. The exhaust air at the same time was incinerated by passing it through the air supply of a lighted, wide-mouthed Fisher gas burner. At the end of atomization the animals were allowed to breathe the infectious cloud for another twenty minutes. The atomizer was then removed and weighed to determine the number of grams of lung virus suspension which had been nebulized.

In different experiments the quantity or amount by weight of lung virus suspension atomized was approximately the same, and the amount of virus inhaled by the mice was regulated by employing ten-fold dilutions of the 10 per cent stock virus material. The amount of virus inhaled will be referred to as lethal air-borne doses (LAD_{50}). Under the conditions of atomization and the time of exposure to the virus cloud, a lethal air-borne dose is considered to be the highest dilution of mouse-lung virus suspension which, when nebulized, will kill half the exposed animals. In general, it may be stated that one LAD_{50} is the amount of virus the mice would inhale following the atomization of 5 ml. of a 10^{-5} dilution of stock 10 per cent mouse-lung suspension over a forty-minute period.

Following each experiment the cloud chamber was washed with O-Syl* and dried with 70 per cent alcohol. The wire cages were washed in O-Syl, rinsed in tap water, and allowed to air-dry. The atomizer was rinsed thoroughly with distilled water and dried with alcohol and ether.

After exposure in the cloud chamber the animals were placed in small metal cages in groups of ten to twelve. The number of mice in different experiments varied, but ten always were set aside for observation concerning the time and rate of death.

Sampling Procedures for Detection of Virus in the Blood, Spleen, Liver, and Lung.—After the lightly etherized mouse was pinned to a cork board, and its fur disinfected with 70 per cent alcohol, the skin was slit from the navel to the throat. The incision was then washed with sterile 0.85 per cent NaCl solution to remove the excess alcohol, and with sterile instruments a cul-de-sac was made in the left axillary region between the skin and the thoracic wall. The axillary artery and vein were then cut. As the blood flowed into the cul-de-sac it was collected with a sterile capillary pipette which had been rinsed in sterile heparin solution (2 mg. heparin sodium per milliliter) and transferred to a sterile test tube kept in an ice bath. Except where especially noted in certain experiments, the blood samples were frozen by shelling in dry ice-alcohol mixture and stored at –50° C.

After the animal had been bled, the entire spleen and roughly half the liver were removed separately under aseptic conditions with separate instruments, care being taken not to puncture the diaphragm. Each was then ground separately with alundum in approximately 1 ml. of sterile antibiotic broth after which the suspensions were frozen and kept at –50° C. until ready for testing for the presence of virus.

The lungs were always removed last with sterile instruments, weighed, and ground with alundum in antibiotic broth to make a 10 per cent suspension which was then frozen as described previously. In some experiments the right upper lobe was removed and prepared for microscopic study.

Detection of Virus in the Samples of Blood, Spleen, Liver, and Lung.—The fertile egg technique was employed for the detection of virus. The samples of blood, and the supernates of the spleen and liver and lung were inoculated separately into the allantoic cavity of 10- to 11-day-old chick embryos. After forty-eight hours' incubation at 37° C., the embryos were chilled overnight at 4° C., and the allantoic fluid of each egg was examined for the presence of virus by hemagglutination of chicken red cells on a spot plate.[4] Usually two to three eggs were inoculated with each sample. Negative fluids from this same sample were pooled and from 0.5 to 0.7 ml. again passed in eggs in the previous manner. If the second passage allantoic fluid showed no hemagglutination, the sample was considered to have contained no virus.

The amount of virus in the lung was determined by first preparing tenfold dilutions of the 10 per cent suspension after which 0.1 ml. of each dilution was inoculated into the chick embryos. The dilution infecting half of the eggs (EID_{50}) was calculated by

*Disinfectant manufactured by Lehn and Fink Products Corporation, Bloomfield, N. J.

the method of Reed and Muench.[5] The LD_{50} virus titer of the lungs was determined by inoculating intranasally groups of three mice under light ether anesthesia with tenfold dilutions of the mouse-lung suspension.

RESULTS

Pathogenesis and Pathology.—The infecting air-borne doses of virus employed were all lethal. Mice exposed to 10 LAD_{50}, however, died from five to eight days after exposure, whereas those allowed to breathe 1,000 LAD_{50} died from three to five days thereafter. The rate of growth of virus in the lungs is approximately the same with the maximum EID_{50} titers of 8.5 to 9.8 being found at forty-eight hours. At this time, however, there was little gross evidence of pulmonary involvement, whereas microscopically the bronchial lining cells showed marked degenerative changes. There were extensive focal areas of inflammation characterized by cellular exudate and edema fluid in the pulmonary spaces. After this time the inflammatory process progressed rapidly so that extensive pulmonary consolidation could be seen grossly at the time of death. Microscopically, such lungs showed marked destruction of the bronchial lining cells with extensive pneumonic consolidation of the lungs as has been described previously.[3]

Influenza Virus in Pooled Blood and Lungs.—The first blood samples analyzed for virus consisted of pooled blood from groups of twenty mice sacrificed at increasing intervals after infections. It was assumed that the amount of virus in the blood at any one time would be small. Thus, an attempt was made to concentrate it by centrifuging the thawed samples at 40,000 r.p.m. in a Spinco* for one hour, after which the supernatant fluid was discarded. The sediment was then resuspended in 3 ml. of antibiotic broth and equal amounts inoculated into five eggs. The results of seven experiments during which the blood was handled in this way are shown in Table I. In the first experiment, none of three samples taken at one, six, and twelve hours after infection was positive for virus while, in the second, two of the six blood samples taken at forty-eight and seventy-two hours after infection contained virus. In only one experiment (No. 3) was virus found in the blood earlier than twenty-four hours. In two of five experiments the twenty-four-hour sample was positive while six of six blood samples taken at forty-eight hours after infection showed virus. In two of three samples collected at seventy-two hours, virus was also present.

In Table I are also shown the virus titers in the pooled lungs of mice from which the blood was taken. It can be seen that, in spite of the high concentration of virus in the lungs at twenty-four hours and later, virus was not isolated from many of the blood samples.

A variety of experiments, during which the blood from individual mice was handled in different ways, were carried out in an attempt to increase the recovery of virus. Because the highest percentage of positive cultures had been obtained from the pooled blood samples collected forty-eight hours after onset of infection (Table I), this interval was employed for sacrificing the

*Beckman Instrument Company, Fullerton, Calif.

animals. The results are shown in Table II. On the basis of these and the previous findings listed in Table I, it appeared that centrifugation of frozen blood samples, increasing the period of egg incubation after inoculation, the use of citrate as an anticoagulant, the addition of chick red cells to the harvested allantoic fluid, or the inoculation of whole blood without a coagulant did not increase the recovery of virus when compared with the number of virus isolations obtained from heparinized, frozen, uncentrifuged blood. In subsequent experiments the latter procedure was generally employed.

TABLE I. INFLUENZA VIRUS IN POOLED BLOOD AND LUNGS OF MICE AT INCREASING INTERVALS AFTER INFECTION WITH 1,000 LAD

EXPER. NO.	TISSUE	TIME AFTER INFECTION IN HOURS						
		1	6	12	24	36	48	72
1	Blood*	Neg.	Neg.	Neg.				
2	Blood	Neg.	Neg.	Neg.				
3	Lung†	–	2.7	3.7	7.2	7.5	7.7	7.2
	Blood	–	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.
4	Lung	1.5	3.0	5.0	7.5	–	9.0	7.7
	Blood	Neg.	Neg.	Neg.	Neg.	–	Pos.	Pos.
5	Lung	–	–	–	7.5	–	7.5	–
	Blood	–	–	–	Neg.	–	Pos.	–
6	Lung	–	–	–	7.5	–	8.0	–
	Blood	–	–	–	Pos.	–	Pos.	–
7‡	Lung	6.0	9.0	7.7	8.2	–	8.5	–
	Blood	Neg.	Neg.	Neg.	Neg.	–	Pos.	–

*Pooled blood of 20 mice.
†Negative log of dilution producing 50 per cent infectivity in eggs.
‡Mice infected intranasally with 200,000 LD_{50} doses. Pooled blood from 10 mice tested.

TABLE II. THE EFFECT OF VARIATION IN TECHNIQUE OF HANDLING BLOOD UPON THE INCIDENCE OF VIREMIA IN INDIVIDUAL MICE BLED FORTY-EIGHT HOURS AFTER EXPOSURE TO 1,000 LAD OF INFLUENZA VIRUS

EXPER. NO.	PROCEDURES	LUNG TITER EID_{50}	NO. POSITIVE / NO. MICE	PER CENT POSITIVE
1.	A. Blood heparinized, frozen, centrifuged at 40,000 r.p.m.		3/10	30
	B. Blood heparinized, frozen (not centrifuged)	8.5	2/10	20
	C. Blood heparinized, frozen, eggs incubated three days		0/10	0
2.	A. Blood heparinized, frozen	8.5	6/12	50
	B. Blood citrated, frozen		6/11	54
3.	A. Blood heparinized, frozen		2/10	20
	B. Blood heparinized, frozen, but allantoic fluid mixed with chick red cells	9.0	2/10	20
4.	A. Blood heparinized, frozen	–	2/10	20
	B. Whole blood without anticoagulant inoculated directly into eggs		0/10	0
5.	A. Heparinized blood, frozen	–	4/10	40
	B. Heparinized blood, frozen	9.0	3/10	30
	C. Heparinized blood, frozen	8.5	5/10	50

Viremia in Individual Mice.—Duplicate sets of experiments were performed to determine whether the size of the infecting air-borne dose of virus effected the incidence of viremia in individual mice. The results are shown in Table III. Only five of 120 mice exposed to the smallest (10 LAD) infecting dose of virus yielded positive blood samples. These were obtained

from five of the twenty animals killed at the fourth day after exposure to the virus cloud. Four of sixty mice yielded virus following exposure to 100 LAD, while the blood of nine of sixty mice infected with 1,000 LAD was positive. These data suggest that the higher infecting doses result in the greater incidence of viremia. However, in spite of the overwhelming infections produced by 1,000 LAD, in only one in six animals was virus demonstrated (Table III).

TABLE III. THE INCIDENCE OF VIREMIA IN INDIVIDUAL MICE IN RELATION TO THE INFECTING DOSE OF AEROSOLIZED INFLUENZA VIRUS

EXPER. NO.	DOSAGE IN LAD	NUMBER MICE WITH VIREMIA / TOTAL NUMBER OF MICE (AT TIME IN HOURS)					
		24	48	72	96	120	144
1	10	0/10	0/10	0/10	1/10	0/10	0/10
2	10	0/10	0/10	0/10	4/10	0/10	0/10
3	100	1/10	0/10	1/10	1/10	All dead	
4	100	-	0/10	1/10	-	All dead	
5	1,000	2/10	2/10	1/10	All dead		
6	1,000	0/10	1/10	3/10	All dead		

TABLE IV. INFLUENZA VIRUS ISOLATION FROM BLOOD, LIVER, AND SPLEEN OF INDIVIDUAL MICE KILLED AT INCREASING INTERVALS AFTER INFECTION

EXPER. NO.	AIR-BORNE DOSES OF VIRUS	TIME AFTER INFECTION (HOURS)	NO. CONTAINING VIRUS IN NO. INDIVIDUAL MICE				NO. CONTAINING VIRUS IN ONE OR MORE TISSUES NO. OF INDIVIDUAL MICE
			BLOOD CELLS	PLASMA	LIVER	SPLEEN	
1	100	48	0/10*	-	1/10	0/10	1/10
		72	1/10*	-	1/10	2/10	3/10
2	1,000	24	2/20	0/20	3/20	4/20	6/20
		48	3/20	0/20	1/20	7/20	9/20
		72	4/20	0/20	4/20	3/20	10/20
3	10,000	24	2/10*	-	0/10	0/10	2/10
	LD_{50} intra-	48	2/10*	-	1/10	4/10	5/10
	nasal	72	2/9 *	-	1/9	4/9	6/9

*Whole blood.

For additional evidence of viremia, the liver and spleen of the mouse as well as its blood were then examined for the presence of virus. In the experiment in which the mice were exposed to 1,000 LAD, the blood was separated into cells and plasma before virus isolation was attempted. The results are shown in Table IV. In some mice all three tissues yielded virus while in others only two, one, or, more frequently, none of the tissues was positive. When the results of these experiments were tabulated to show the number of different mice from which virus was isolated by analysis of these organs, the number showing evidence of having, or having had, a viremia was significantly increased. For instance, only four of the twenty mice exposed to 1,000 LAD (Experiment No. 2) and killed seventy-two hours after infection had virus in the blood, while six additional mice with negative blood cultures showed virus either in their livers or spleens or both. Thus, ten of the twenty mice on the basis of virus isolation from the three tissues showed evidence of a viremia. A similar increase in incidence of viremia was also obtained by analysis of the livers and spleens in addition to the blood of mice infected by

the air-borne or intranasal methods and killed at earlier intervals after infection (Table IV). It was of interest to note that the direct intranasal instillation of the virus did not result in a greater incidence of viremia compared with findings in animals infected by allowing them to breathe nebulized virus.

TABLE V. INFLUENZA VIRUS ISOLATION FROM THE BLOOD OF MICE INFECTED WITH 1,000 LAD AND BLED REPEATEDLY FROM THE TAIL VEIN

EXPER. NO.	TOTAL NO.	MICE			TIME OF VIREMIA IN POSITIVE MICE							
		NO. NEG.	NO. POS.	PER CENT POS.	BLOOD SAMPLE TAKEN AT HOUR	MOUSE NUMBER						
						1	2	3	4	5	6	7
1.	20	17	3	15	24	Neg.	+	Neg.				
					48	+	Neg.	+				
					72	Neg.	Neg.	Neg.				
2.	10	7	3	30	19	Neg.	Neg.	Neg.				
					24	Neg.	Neg.	Neg.				
					44	Neg.	Neg.	+				
					48	Neg.	+	Neg.				
					66	+	Neg.	Neg.				
3.	10	8	2	20	44	Neg.	Neg.					
					45	+	Neg.					
					46	Neg.	Neg.					
					47	Neg.	Neg.					
					48	Neg.	+					
4.	15	11	4	26	47	Neg.	Neg.	Neg.	Neg.			
					48	Neg.	Neg.	+	+			
					49	Neg.	+	+	Neg.			
					50	Neg.	Neg.	+	Neg.			
					51	+	Neg.	Dead	Neg.			
5.	20	13	7	35	47	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
					48	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	+
					49	Neg.	Neg.	Neg.	+	Neg.	+	Neg.
					50	Neg.	Neg.	Neg.	+	Neg.	Neg.	Neg.
					52	+	+	+	+	+	Neg.	Neg.

Duration of Viremia in Individual Mice.—In spite of the sporadic isolation of influenza virus from pooled and individual bloods of mice sacrificed at increasing intervals after onset of fatal influenza infections, an attempt was made to isolate virus from the blood of individual mice repeatedly bled following exposure to nebulized virus. From 0.05 to 0.2 ml. of blood was collected from the tail vein in a white blood cell-counting pipette which had been previously rinsed in sterile heparin and dried. Each sample was mixed with approximately 0.25 ml. of antibiotic broth after which it was frozen. When ready for testing each sample was inoculated intra-allantoically into one egg. The result of five such experiments are recorded in Table V. As was observed with pooled and individual blood, only from 15 to 35 per cent of the mice yielded samples of blood which were positive for influenza virus. In most instances only one of several blood samples from individual mice was positive. However, three of four samples of blood from one mouse in Experiment No. 4 and three of five samples from a mouse in Experiment No. 5 yielded virus. In an additional experiment not shown in Table V ten mice were each bled from the tail every four hours starting thirty-six hours after infection with 1,000 LAD of influenza virus for a period of another thirty-six hours. No virus was isolated from any of the samples of blood.

Distribution of Virus in the Blood.—In Experiment No. 2 shown in Table IV no virus was isolated from the plasma whereas nine of sixty mice showed virus in the cell portion of the blood. Additional experiments were then carried out to gain further information concerning the distribution of virus in the different fractions (red cells, white cells, and plasma) of the blood. Pooled heparinized blood of twenty mice killed forty-eight hours after infection with 1,000 LAD was employed. It was collected in silicon*-coated glassware to prevent injury to the white blood cells and centrifuged at 2,000 r.p.m. The plasma, white cells, and red cells were removed, respectively, and frozen until ready for virus analysis. On the day of egg inoculation buffered saline solution was added to the red and white cell fractions after thawing after which these, along with the plasma fraction, were subjected to high-speed centrifugation at 40,000 r.p.m. in the Spinco for one hour. The sediments were then separately injected into groups of five eggs. The results are shown in Table VI.

TABLE VI. DISTRIBUTION OF INFLUENZA VIRUS IN POOLED BLOOD OF MICE FORTY-EIGHT HOURS AFTER INFECTION WITH 1,000 LAD

EXPER. NO.	BLOOD FRACTIONS		
	ERYTHROCYTE	LEUKOCYTE	PLASMA
1.	+	Neg.	Neg.
2.	+	Neg.	Neg.
3.	+	Neg.	Neg.
4.	+	Neg.	Neg.
5.	+	Neg.	+
6.	+	Neg.	+
7.	+*	- - -	Neg.
8.	+*	+	Neg.
9.	+	+	+

*Virus not isolated from saline washings of erythrocytes.

In all nine experiments listed in Table VI influenza virus was demonstrated in the red cell fraction. In two of eight analyses it was also present in the white cell fraction while three of nine also yielded virus in the plasma fraction. In only one experiment (Experiment No. 9) was virus demonstrated in all three fractions. In Experiments Nos. 7 and 8 the erythrocytes were not frozen but were immediately washed in buffered saline solution after which the washings were concentrated in the Spinco. In spite of the washings the red cells yielded virus, whereas the saline washings did not.

To test the possibility that the failure to isolate virus from the plasma fraction might be due to nonspecific virus-neutralizing substances, as suggested by Ginsberg and Horsfall[16] and by Bang and associates,[17] the following experiments were carried out. A small quantity (approximately 10 EID_{50}) of influenza virus was added to heparinized mouse blood in vitro and the mixture was incubated at 37° C. for twenty minutes. It was then centrifuged at low speed to separate the blood components. Each fraction was then analyzed for virus as described previously. In two such experiments virus was isolated both from the red cell and plasma fractions and once from the leukocyte fraction.

*Silicon compound Drifilm 9987, General Electric Company, Schenectady, N. Y.

Under the conditions of these experiments the plasma did not contain sufficient virus-neutralizing substances to prevent the re-isolation of the added virus.

Recovery of Virus From the Blood, Spleen, and Lungs After Intravenous Infection.—The various procedures thus far carried out to demonstrate viremia in experimental pulmonary infections in mice indicated that it was sporadic and of short duration and that only a small amount of virus was present at any one time. The following experiments, listed in Table VII, were carried out to determine the duration of viremia when influenza virus was inoculated directly into the blood stream. Each mouse was inoculated via the tail vein with 0.1 ml. of diluted virus equivalent to 5,000 EID_{50} (approximately 100 LD_{50} intranasal doses). Sufficient numbers of mice were inoculated so that groups of five could be sacrificed at close intervals of time as shown in Table VII. In

TABLE VII. INFLUENZA VIRUS ISOLATION FROM THE BLOOD, SPLEEN, AND LUNG AFTER INTRAVENOUS INJECTION

EXPER. NO.	TIME AFTER INJECTION	NUMBER OF MICE OF A TOTAL OF FIVE SHOWING VIRUS IN SAMPLES OF: BLOOD	SPLEEN	LUNG
1.	1 min.	5	4	5
2.	5 min.	4	3	5
1.	8 min.	1	4	5
2.	10 min.	1	1	4
1.	15 min.	0	4	2
2.	20 min.	2	0	5
1.	30 min.	0	0	2
1.	1 hr.	0	0	3
2.	1 hr.	0	1	4
1.	6 hr.	0	0	1
2.	6 hr.	0	1	2
1.	8 hr.	0	0	1
2.	12 hr.	0	0	0
2.	24 hr.	0	0	1
2.	48 hr.	0	0	0
2.	72 hr.	nd	nd	0
2.	96 hr.	nd	nd	0

spite of the large amount of virus given, it was not isolated from the blood thirty minutes or longer after inoculation. In the case of the spleen, virus was consistently isolated from some of the animals killed up to fifteen minutes after inoculation. Thereafter, virus was isolated from the spleen of only one of five animals killed at one and six hours after inoculation, respectively. In these experiments influenza virus was most frequently recovered from the lungs as shown by the fact that this organ yielded virus in some of the animals killed up to eight hours after inoculation. Thereafter, the lungs of only one of five animals killed at twenty-four hours was positive.

DISCUSSION

A number of published investigations has been concerned with the problem of viremia in experimental influenza virus infections in animals with variable results. In the earlier studies identification of the virus was made by inoculating the blood and suspensions of ground tissues intranasally into mice or ferrets.

In the more recent reports the fertile egg technique was employed. During their initial studies on the pathogenesis of experimental influenza A virus (WS strain) pulmonary infections in ferrets and mice, Smith, Laidlaw, and Andrewes[6-8] were unable to demonstrate virus in the blood, spinal fluid, liver, spleen, or brain of animals killed at the height of the disease, whereas, at this time, it could be isolated readily from the tissues of the respiratory tract. They could not demonstrate evidence of infections with the influenza virus when it was inoculated subcutaneously or intraperitoneally. Orcutt and Shope[9] failed to demonstrate swine influenza virus in the blood, spleen, liver, kidney, mesenteric lymph nodes, colon mucosa, or brain of infected swine which were sacrificed three and four days after intranasal inoculation of virus. In such animals virus was found only in the tracheal exudate, turbinates, and bronchial lymph nodes.

Cerruti,[10] employing the WS strain, was able only to isolate virus from the blood of intranasally infected mice by serially passing the lungs of mice into which the blood was originally inoculated intranasally. As in the English studies, no visible lesions or evidence of infection could be seen in the mice when the virus was given subcutaneously or intraperitoneally. Employing the mouse for identification of virus, Smorodintseff and Ostrovskaya[11] were able to detect small amounts of influenza virus in the blood, urine, bile, spleen, and liver of mice infected intranasally with a lethal dose of WS or Leningrad virus. Again, in many animals they were able only to demonstrate virus in the extra pulmonary tissues by serial passage of the lungs into which the material was first inoculated. They also showed that when large amounts of virus were inoculated intravenously or intraperitoneally, it rapidly disappeared and did not localize in the lungs as indicated by the development of pulmonary lesions. Rickard and Francis,[12] on the other hand, demonstrated the localization of large amounts of PR8 virus in the lungs of mice when administered intravenously or intraperitoneally. In spite of the persistence of virus in the lungs in high titer for several days, at no time after inoculation was virus demonstrated in the blood.

Bijl and associate[13] demonstrated virus in the blood, spleen, liver, brain, kidney, and adrenal of many mice killed forty-eight hours after the intranasal inoculation of influenza virus. Blood diluted as much as 1:500 was found to contain virus by inoculating this material intranasally into normal mice. They also found that following the intraperitoneal inoculation of large amounts of virus in mice it could no longer be demonstrated in the organs or blood after the second day.

Only two studies have been reported during which the fertile egg technique was employed for demonstrating the influenza virus in extrapulmonary tissues. Burnet and Lush[14] detected small amounts of influenza virus in the spleens of mice killed on the second and third days following intranasal inoculation of the virus. Fauconnier and Beauchamp[15] reported incidences of viremia in intranasally infected mice (PR8 strain) varying from 12 to 56 per cent over

the period from twelve hours postinfection to 103 hours thereafter. They were unable to correlate any change in the incidence of viremia in relation to time after onset of infection. Our studies, in general, support those of the French workers. In our experiments in which the mice were allowed to breathe highly lethal clouds of influenza virus the incidence of viremia ranged from 20 to 50 per cent, and there was little evidence that the incidence changed with time. Virus was found in the pooled or individual bloods of mice in general only when the virus content of the lung was high. On the other hand, in many animals with equally high lung-virus titers, no virus could be demonstrated in the blood.

The results of this study, as well as those which have been reported by others, indicate that the amount of virus which passes from the lung into the blood in experimentally infected mice is small and near the limits of detection with the fertile egg technique. Only 12 per cent of bloods from mice infected with highly lethal clouds of influenza virus (1,000 LAD) and killed from twenty-four to seventy-two hours thereafter yielded virus on the first egg passage. On the second egg passage of the negative first passage allantoic fluids, an additional 16 per cent were found to contain virus. A further significant increase in the percentage of animals showing evidence of a viremia was obtained by analysis of the livers and spleens of the mice. It was assumed that the influenza virus found in these organs was transported there from the lungs via the blood stream.

It would also appear from these studies that viremia is either sporadic or that once outside the lungs the virus becomes rapidly inactivated. Whether failure to isolate virus from the blood in a higher percentage of mice was due to "non-specific virus neutralizing substances" described by Ginsberg and Horsfall[16] and Bang and associates[17] was not determined. Failure to isolate virus from many samples from serial bleedings of individual mice was undoubtedly due to the small amount of blood and the failure of the virus present to withstand the freezing and thawing procedure which preceded egg inoculation.

Of interest in this study was the observation that the influenza virus in the blood was found in the erythrocyte fraction and that it was actually attached to the erythrocyte. However, there was not sufficient virus present to produce gross or microscopic agglutination of red cells. Should the virus-coated erythrocytes become agglutinated in vivo, they would be expected to be filtered out more quickly in the vascular beds of the liver, spleen, and lung. This, then, could account for the rapid disappearance of influenza virus from the blood and its localization in the liver, spleen, and lung, as observed in this and other studies.[12] The rapid disappearance of influenza virus from the blood stream and its persistence in the lung, spleen, and so on, is similar to the behavior of carbon particles and certain bacteria[18] when injected intravenously and also similar to the behavior in intravenously injected labeled antigen.[19] Thus, the persistence of influenza virus in the lung following intravenous injec-

tion may be accounted for as a result of the effective filtering capacity of this organ and not necessarily because of the "pneumotropic" character of the virus.

The association of viruses in vivo with the blood cells rather than the plasma has been noted previously for vaccinia, where the specific cell was the leukocyte, and fowl plague where both leukocytes and erythrocytes have been implicated.[18] Failure to isolate consistently influenza virus from leukocytes cannot be regarded as evidence that phagocytosis of the virus does not occur, since it is possible that rapid inactivation of the virus may take place after ingestion by leukocytes. However, the recent reports by Boand and associates[20] indicate that in vitro influenza virus is not readily phagocytosed by normal rabbit leukocytes, although it is taken up by phagocytes from immune animals, or by normal leukocytes in the presence of immune serum.

The role of viremia in the pathogenesis of pulmonary influenza virus A infections in mice needs further evaluation. Certainly it is not essential for the development of pulmonary lesions following intranasal inoculation of the virus. It may represent simply an intermittent "spilling over" of virus from the lung after the lung-virus titer becomes high and the inflammatory response pronounced. Viremia, however, is probably essential for eliciting a good generalized antibody response since it has been shown that passive circulating antibody suppresses completely the development of detectable antibody in animals with obvious pulmonary infections.[1] The logical explanation of this observation is, on the basis of this study, that the passive circulating antibody immediately neutralizes the virus after it enters the blood stream and prevents it from stimulating a generalized immune response on the part of the reticuloendothelial system of the liver, spleen, and other extrapulmonary tissues. Whether viremia occurs in natural influenza infections in man is not known. It probably does in some instances and may account for some of the findings, toxic manifestations, and antibody response to the disease.

SUMMARY

Viremia occurs in mice infected intranasally or in the cloud chamber with lethal doses of influenza A (PR8 virus). The incidence of viremia in mice killed at forty-eight hours after infection varied from 20 to 50 per cent in mice allowed to breathe highly lethal clouds of nebulized virus. With few exceptions viremia occurred only in animals after the concentration of influenza virus in the lungs had reached a high level. On the other hand, virus was not isolated from the blood of many animals with similar lung titers so that the incidence of viremia could not be correlated with the amount of virus or the pathologic findings in the lungs.

Viremia in experimental pulmonary infections in mice is probably sporadic with the amount of virus in the blood at any one time being small and close to the minimal infecting dose in fertile eggs when inoculated intra-allantoically. The duration of viremia in individual mice could not be determined accurately because of the small amount of blood available for virus analysis. When influenza virus enters the blood from the lungs it quickly becomes attached to

the red blood cells. Whether the virus-coated cells become agglutinated in vivo was not determined. Following the intravenous injection of influenza virus it rapidly disappears from the blood but can be detected for significantly longer periods in the spleen, liver, and lung. The possible significance of viremia in relation to the pathogenesis and immune response in experimental infections in mice is discussed.

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PRELIMINARY CHARACTERIZATION OF A GROUP OF UNIDENTIFIED PLEOMORPHIC BACTERIA ISOLATED FROM BLOOD AND OTHER TISSUES

CHARLES M. CARPENTER, M.D., DEXTER H. HOWARD, PH.D., AND ELSIE L. LEHMAN, B.A.
LOS ANGELES, CALIF.

UNIDENTIFIED pleomorphic bacteria which form minute colonies on enriched agar plates have been isolated repeatedly from the blood of patients with chronic illness. For the most part, such illnesses included fevers of undetermined origin, Hodgkin's disease, leukemia, lymphosarcoma, chronic brucellosis, and cardiovascular disease.[3] This report deals with the results obtained from a study of fifty strains of these organisms, which have been designated as unidentified pleomorphic bacteria (U.P.B.), until their taxonomic relationship to other bacterial species has been ascertained. Forty-four strains were isolated from blood of 100 patients with prolonged illness, two strains from bone marrow, and one strain each from biopsied muscle tissue, synovial fluid, urine, and a lymph node.

MATERIAL AND METHODS

Blood Cultures.—Twenty milliliters of blood was obtained from the patient by venipuncture, after careful disinfection of the skin with iodine and alcohol. Five milliliters of blood was distributed to each of the following: (1) a flask containing 75 ml. of tryptose-phosphate broth (Difco), (2) a flask containing 75 ml. of trypticase-soy broth (BBL), (3) a two ounce bottle containing a slant of brucella agar (Albimi), supplemented with thiamin, nicotinamide, and hemin, as recommended by Liberman and Pickett[14] for the cultivation of brucellae. The base of this agar slant was immersed in 10 ml. of brucella broth (Albimi), supplemented as above, in accordance with the Castaneda procedure,[4] (4) a screw-cap tube containing 20 ml. of distilled water.

The three media receiving whole blood were incubated at 36.5° C. in an atmosphere of 10 per cent carbon dioxide. They were subcultured on brucella agar, supplemented as before, on the third, sixth, ninth, fourteenth, and twenty-first days of incubation. The lysed blood in distilled water was centrifuged at 3,000 r.p.m. for 30 minutes and the supernatant was discarded. One-tenth milliliter aliquots of the remaining blood sediment were inoculated onto brucella agar, supplemented as before, human blood agar, and chocolate agar, all of which were incubated at 36.5° C. in an atmosphere of 10 per cent carbon dioxide. One-tenth milliliter of the blood sediment was inoculated into thioglycollate broth (Difco). Subcultures of the thioglycollate broth were made as they were with the media receiving whole blood and incubated under anaerobic conditions.

From the Department of Infectious Diseases, School of Medicine, University of California, Los Angeles, and Investigative Medical Service, Veteran's Administration Hospital, Long Beach, Calif.

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A series of eight blood cultures was made from 35 of 100 patients, 10 were cultured either two or three times, and a single blood culture was obtained on the remaining 55 patients. In those instances in which a series of eight blood cultures was obtained, four were taken on a single day at two-hour intervals and one daily for four days.

Other Cultures.—Bone marrow for culture was obtained aseptically from either the sternum or the iliac crest.

Tryptose-phosphate and trypticase-soy broths were not employed because of the limited amount of inoculum. Five-tenths milliliters of marrow was inoculated in the Castaneda medium described above. The remaining material was distributed to each of the following: (1) one plate each of human blood agar, chocolate agar, and brucella agar supplemented as before, (2) a tube of thioglycollate broth. The media were incubated and subcultured as in the case of the cultures on blood.

Synovial fluid was obtained from the knee joint in the usual manner. The material was cultured in the same manner as it was for the blood, except for the omission of the distilled-water lysing procedure. All incubations and subcultures were carried out as for the blood cultures.

The muscle-biopsy specimen and lymph node were first ground in a sterile mortar and pestle and then extracted with saline. The saline extract was cultured in the same manner as the synovial fluid.

The urine was first concentrated by centrifugation. The sediment was then cultured on human blood agar, chocolate agar, and brucella agar, supplemented as before, and in the Castaneda medium. Incubation and subcultures were carried out as with the blood cultures.

Routine Cultures.—All strains of U.P.B. isolated were maintained at 5° C. on slants of brucella agar supplemented as before and were transferred every six weeks. All cultures, unless otherwise indicated, were incubated at 36.5° C.

Cultural and Microscopic Morphology.—Colonial morphology was obsreved at 24, 48, and 72 hours and at one week with a dissecting microscope (×30), with the employment of both directly and obliquely transmitted light. Organisms were stained by Gram's method, with Loeffler's methylene blue, and by the Ziehl-Neelsen method for acid-fast organisms. Tests for motility were carried out by inoculation of cultures into cystine trypticase agar (BBL) and the recording of results at 24 and 48 hours.

Carbohydrate Fermentations.—Sufficient carbohydrate to yield one per cent concentrations was added to 3 ml. of cystine trypticase agar. The carbohydrate agar was sterilized by autoclaving. Cultures on this medium were observed for acid production after 24, 48, and 72 hours', and one week's incubation.

Gelatin Liquefaction.—The cultures were streaked across plates of brucella agar containing 0.5 per cent gelatin. After 48 hours incubation, the plates were flooded with 8 to 10 ml. of the following solution: $HgCl_2$, 15 Gm.; concentrated HCl, 20 ml.; distilled water, 100 ml. Absence of a white precipitate surrounding a colony indicated a positive test.[10]

Other biochemical tests included in this report were performed in accordance with the procedure recommended in the Difco Manual.[6]

Antibiotic Sensitivity.—Tests for sensitivity to antibiotics were made with Sensitivity Discs (Difco) placed on preinoculated plates of brucella agar. Observations were made after 24 and 48 hours' incubation. The degree of sensitivity or resistance to penicillin was further evaluated by use of brucella agar plates to which had been added sufficient sterile crystalline-G penicillin to yield levels of 1, 10, 100, and 1,000 units per milliliter of the medium.

Studies on Pathogenicity for Chick Embryos and Laboratory Animals.—Pathogenicity of the U.P.B. for five-day-old chick embryos was determined by yolk sac inoculation with 0.1 ml. of 48-hour broth cultures. For each strain, five eggs were employed, four of which received 0.1 ml. of the undiluted broth culture and one egg was inoculated with the same amount of a 1:10 dilution. The eggs were placed in an incubator, candled daily, and

upon death of the embryo, yolk sac cultures were made. Twenty controls were employed, 10 of which received yolk sac inoculations of 0.5 ml. plain brucella broth and 10 of which were normal unopened eggs. Yolk sac cultures of six live eggs were made on the eighth day of incubation. Suckling mice, mature mice, rats, guinea pigs, hamsters, and rabbits received 0.5 ml. of the same inoculum, intraperitoneally, intravenously, intramuscularly, intracerebrally, intranasally, and onto the conjunctiva. At autopsy, both blood and tissue cultures were made on brucella agar.

RESULTS

Microscopic Morphology.—The unidentified pleomorphic bacteria are nonmotile, nonencapsulated, nonspore-forming, nonacid-fast rods, which show club forms, palisading, and metachromatic granules and bars, resembling

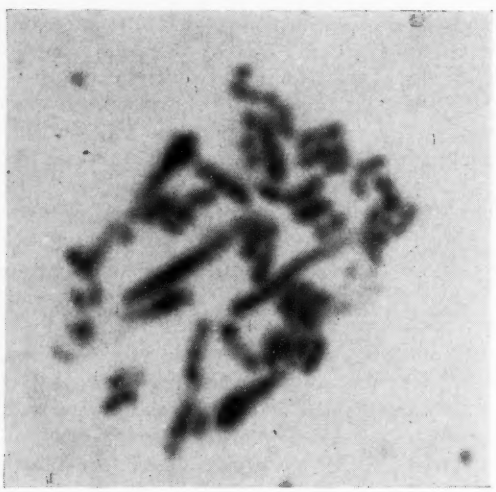

Fig. 1.—Gram stain of a typical strain of U.P.B. (Approximate magnification ×2000.)

corynebacteria. Bacillary forms are slender, straight, curved, and occasionally in ring form. They stain irregularly with methylene blue, showing bars and beading within the rod and often simulating a shortchained *Streptococcus.* Many strains show club formation. They may occur singly or in bi-polar formation or they may form acute or obtuse angles. Cellular arrangements suggesting branching have been observed. In general, the organisms are gram-variable, although granules, bars, and clubs are consistently gram-positive. Certain strains tend to hold the stain more tenaciously than others.

In 24-hour cultures, coccoid, coccobacillary or lancellate forms frequently predominate, clubs and rods being less evident. Any number of bizarre forms may be present. Coccoid forms range from 0.2 to 1μ in diameter, and bacillary forms vary in length from 0.5 to 3μ, with occasional filamentous forms reaching extreme lengths. The great variation in morphology exists not only among different strains but also within a given strain. The predominating morphology depends upon the age of the colony, the media upon which it was grown, and the conditions of incubation. Fig. 1 presents some of the microscopic features discussed above.

Colonial Morphology.—Little variation occurs in colonial morphology on different media. The typical young colony, incubated 48 hours on brucella agar, is minute, varying from 0.1 to 0.3 mm. in diameter. It is round, with an

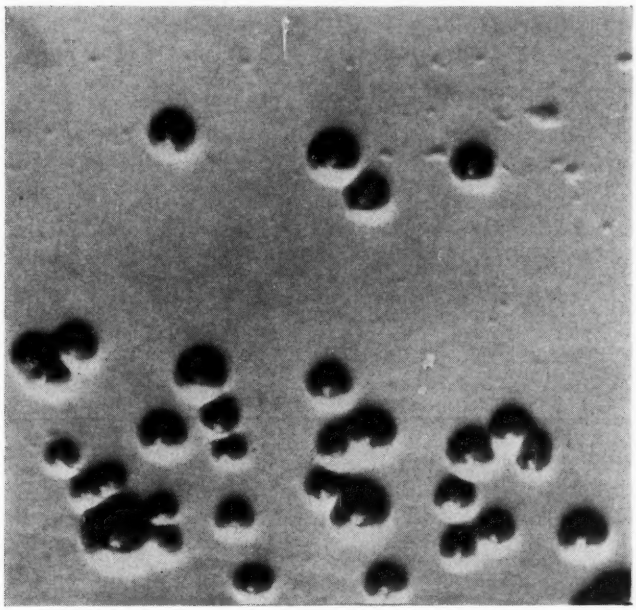

Fig. 2.—Colonies of U.P.B. on the surface of agar. Forty-eight hours' incubation, 36.5° C. (Approximate magnification ×30.)

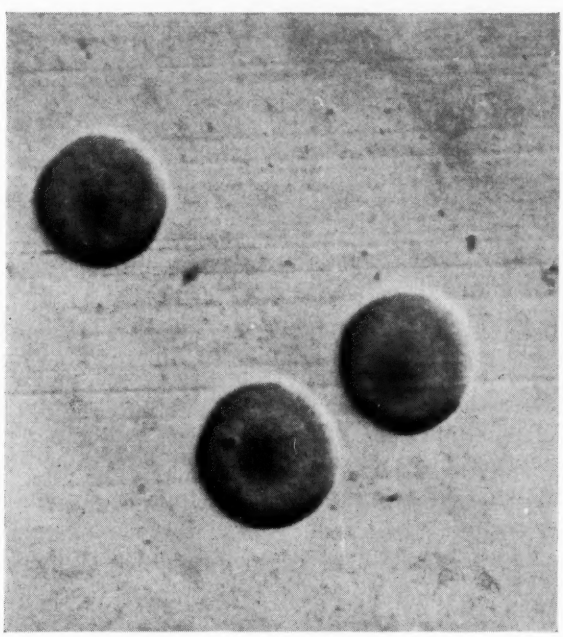

Fig. 3.—Colonies of U.P.B. on surface of agar plate. Forty-eight hours' incubation 36.5° C. Enlargement of Fig. 2. (Approximate magnification ×90.)

entire or undulate margin, and flat, with a finely granular surface, a papillate center, and an elevated periphery (Figs. 2 and 3). Colonies are opalescent with a bluish cast when observed by obliquely transmitted light. By reflected light they have a light cream appearance, often showing dark brown granules and a darker brown central portion. In about a week the colonies have a crenated appearance, becoming more flattened and developing a punctiform center and a serrated periphery (Fig. 4).

In broth cultures, the U.P.B. produce either a uniform turbidity or a finely granular sediment at the bottom of the culture tube with no turbidity. Pellicle formation has not been observed to occur.

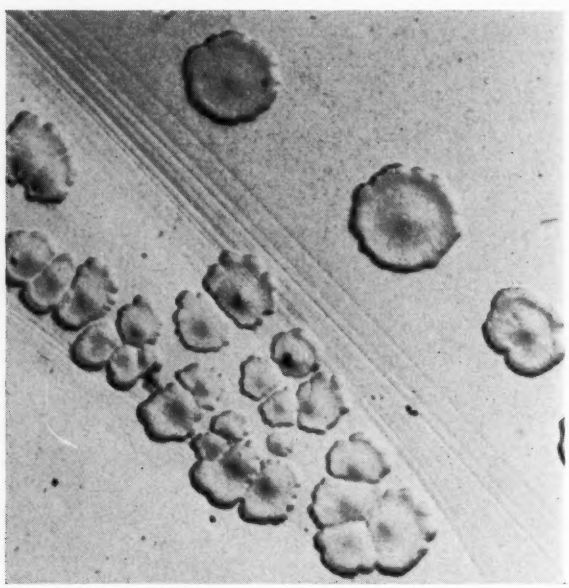

Fig. 4.—Colonies of U.P.B. on surface of agar plate. One week's incubation 36.5° C. (Approximate magnification ×90.)

Growth on human blood agar is satisfactory. Small pinpoint colonies appear in from two to five days, showing no hemolysis. Pigmentation is absent on Loeffler's blood serum (Difco), and the medium is not liquefied. On tellurite blood agar (Difco), most strains produce colonies which are very minute, round, flat, and dry, varying from slate-gray to black in color.

Most strains of unidentified pleomorphic bacteria are aerobes and facultative anaerobes; however, two strains are definitely microaerophylic.

The optimum temperature range for growth is from 34° to 37° C. Satisfactory growth, however, was obtained at room temperature, approximately 23° C., and sixteen strains exhibited slight growth at 4° C. All strains failed to grow after exposure for ten minutes to temperatures ranging from 58° to 60° C.

Biochemical Reactions.—The U.P.B. produce only slight pH changes in the carbohydrate agar used in this study. Nevertheless, strains of U.P.B. were

recorded as giving a positive fermentation wherever color changes could definitely be ascertained. The biochemical reactions tested are summarized in Table I.

Antibiotic Sensitivity.—Table II shows the results of a study of the antibiotic resistance and sensitivity of these organisms. Tableted reagents were used for most antibiotics inasmuch as these are most commonly employed for this purpose in clinical laboratories.

TABLE I. BIOCHEMICAL REACTIONS OF 50 STRAINS OF U.P.B.

BIOCHEMICAL REACTION	NUMBER OF STRAINS (POSITIVE)	NUMBER OF STRAINS (NEGATIVE)
Catalase production	50	0
Gelatin liquefaction	11	39
Hydrogen sulfide production	0	50
Indol production	1	49
Peptonization of milk	7	43
Nitrate reduction	4	46
Urea hydrolysis	1	49
Acetyl-methyl-carbinol production	0	50
Carbohydrate fermentations		
Glucose	32	18
Lactose	22	28
Sucrose	25	25
Maltose	23	27
Galactose	14	36
Fructose	15	35
Dextrin	6	44
Starch	2	48
Mannitol	4	46
Glycerol	1	49

TABLE II. ANTIBIOTIC SENSITIVITY OF 50 STRAINS OF U.P.B.

ANTIBIOTIC AND CONCENTRATION	R*	S†
Penicillin—1 unit per ml. of medium	13	37
Penicillin—10 units per ml. of medium	10	40
Penicillin—100 units per ml. of medium‡	9	41
Penicillin—1,000 units per ml. of medium	7	43
Streptomycin—10 μg (Disc)	9	41
Chlortetracycline—30 μg (Disc)	2	48
Oxytetracycline—30 μg (Disc)	1	49
Erythromycin—10 μg (Disc)	1	49
Tetracycline—100 μg (Disc)	0	50

*Resistant.
†Sensitive.
‡Duplicated in disc.

Animal Pathogenicity.—The unidentified pleomorphic bacteria failed to produce observable disease in any of the laboratory animals, and after infection, they could not be recovered from the blood or tissues of any of the laboratory animals. All strains, however, were pathogenic for 5-day-old chick embryos and caused death in from 1 to 14 days. U.P.B. were recovered from the yolk sac of all infected embryos at the time of death, but in no case could they be recovered from the noninfected control embryos.

DISCUSSION

In view of the marked morphologic similarity of the unidentified pleomorphic bacteria to certain species of corynebacteria, taxonomically they might

be placed within the genus. With the exception of their unique colonial morphology, their biologic characteristics are in many ways typical of the so-called diphtheroid group. Fleisher[9] has described similar organisms as diphtheroids and undoubtedly other bacteriologists have observed them. The literature contains numerous reports of corynebacteria, other than *Corynebacterium diphtheriae,* which have been isolated from man. Bunting and Yates,[2] and Rosenow[18] reported the presence of diphtheroids in cultures from human sources. Subsequently, numerous bacteriologists have reported similar observations.[1, 7, 8, 12, 13, 15, 16] It should be pointed out, however, that most of the organisms heretofore described as diphtheroids, although resembling the U.P.B., produce colonies which are considerably larger, high-convex, with a moist, glistening, and sometimes pigmented surface. Morton reported a dwarf-colony variant of *C. diphtheriae* which, with the exception of exotoxin production, resembles very closely the organisms designated as U.P.B.[17] Such dwarf or gonidial type colonies have also been reported among the micrococci.[11]

Another group of organisms should be considered briefly. The pleuropneumonia-like organisms,[19] although differing markedly biologically, do bear some resemblance morphologically to the U.P.B. group. The biochemical characteristics of the U.P.B. failed to provide a means of classification within the group. It is anticipated that future serologic studies may prove to be a more satisfactory aid in classifying the group.

It should be emphasized that this study is a preliminary survey of a highly variable group of bacteria, which require far more investigation before their clinical significance can be assessed. In view of the consistency with which they have been isolated, it cannot be doubted that U.P.B. are present in the blood and tissues of patients with certain chronic illnesses. At the present time, their etiologic relationship to disease is not established. A number of possibilities, however, should be considered.

The frequency of antibiotic-resistant strains within the group suggests the possibility that they may be antibiotic-induced variants of other bacterial forms. The frequent isolation of U.P.B. concurrently with other typical bacteria lends support to such a hypothesis.[3] It is not inconceivable that after prolonged exposure to chemotherapeutic agents, variants might arise so radically altered morphologically and biologically that their identification is not possible with our present bacteriologic methods. The existence of L-forms, which occur within most genera, is an outstanding example of such a marked variation.[5] It is interesting to note that the two antibiotics to which the greatest number of strains demonstrated resistance, namely penicillin and streptomycin, are the two antibiotics which have been employed extensively for a considerable period of time as chemotherapeutic agents.

If, on the other hand, the U.P.B. constitute a special group of pathogenic organisms, their virulence may depend upon a synergistic or symbiotic relationship with recognized disease producing microorganisms. If such a relationship exists, it would appear that a rather high degree of host specificity may be operative, inasmuch as pathogenicity could not be demonstrated for laboratory

animals. Furthermore, it is possible that the presence of other microorganisms or of certain host factors, such as stress or deranged physiology, are necessary for the U.P.B. to manifest pathogenicity. It is interesting to note that a striking parallel exists between the principal sources of Fleisher's strain and those from which the U.P.B. were obtained.[9] In both instances the majority of strains were recovered from patients with fevers of undetermined origin and from such chronic illnesses as Hodgkin's disease, leukemia, and malignant lymphoma.

SUMMARY

1. Bacteriologic studies were carried out on fifty strains of unidentified pleomorphic bacteria (U.P.B.). They were isolated from the blood and tissues of patients with chronic illness.

2. The U.P.B. are nonmotile, nonspore-bearing, nonacid-fast rods. On solid media they form minute round colonies from 0.1 to 0.3 mm. in diameter. Colonies are flat with an entire edge, a finely granular surface, papillate center, and elevated periphery. Pigmentation is absent. Microscopically, the organisms show marked pleomorphism. They are gram-variable and stain irregularly with methylene blue, showing club forms, palisading, and metachromatic granules and bars resembling corynebacteria.

3. Biochemical reactions were essentially negative, carbohydrates being fermented only very slightly if at all. All of the strains formed catalase and a few strains reduced nitrates to nitrites.

4. Thirteen of 50 strains tested of the U.P.B. were resistant to penicillin, nine to streptomycin, and a few demonstrated resistance to other antibiotics. Only one strain was resistant to erythromycin, one to oxytetracycline, and none to tetracycline.

5. Although none of the U.P.B. were pathogenic for laboratory animals, all were pathogenic for 5-day-old chick embryos and caused death of the embryos in from one day to two weeks.

6. Inasmuch as the variable morphology of the U.P.B. resembles certain species of corynebacteria, taxonomically they may belong to this genus. With the exception of their unique colonial morphology, their biologic characteristics are in many ways typical of the so-called diphtheroid group. It is suggested that the pathogenicity of these bacteria may depend upon a synergistic or symbiotic relationship with other microorganisms or upon a deranged physiologic state in the host.

We are grateful to Doris M. Monroe for technical assistance.

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THE CONCENTRATION OF TETRACYCLINE IN THE SERUM FOLLOWING PARENTERAL ADMINISTRATION

BARTIS KENT, M.D., ELLARD M. YOW, M.D., AND EDNA TOWNSEND, B.A.
HOUSTON, TEX.

CLINICAL trials have revealed that tetracycline has approximately the same therapeutic range but is somewhat better tolerated than oxytetracycline and chlorotetracycline.[1] Its usual mode of administration has been by the oral route. There are certain patients, however, in whom the administration of the antibiotic by this route is not feasible. Therefore, an attempt has been made to determine the level of the antibiotic attained after intramuscular and intravenous administration. Since serum levels necessary for therapeutic effectiveness of tetracycline have not been established and undoubtedly vary with the specific disease or causative organism being treated, no attempt was made to decide whether or not the levels attained were therapeutically significant.

METHOD

The drug used in this study was the Tetracyn brand of tetracycline, produced by the J. B. Roerig Co. Hospitalized adults were selected who were not receiving other antibiotics and represented a cross section of adult patients on the various services of the hospitals participating in the study. The patients were divided into three groups, one group receiving a single intramuscular injection of either 50 mg., 100 mg., 250 mg., or 500 mg. of the drug. The drug was dissolved in distilled water in a concentration of 100 mg. per milliliter and was injected deep into the gluteal muscle of the patient. Blood specimens were then collected at 1-hour, 4-hour, 8-hour, 12-hour, and 24-hour intervals.

A second group of patients received either 50 mg., 100 mg., or 250 mg. injections at eight-hour intervals at 6:00 A.M., 2:00 P.M., and 10:00 P.M. over a five-day period. Daily blood specimens were obtained at 8:00 A.M., or two hours after the 6:00 A.M. injection. The ward nurses were depended on to give the injections at the standard times and an attempt was made to collect the daily specimens exactly two hours after the morning injection; however, this was subject to some minor variations.

A third group of patients was given intravenous injections of either 100 mg., 250 mg., or 500 mg. of tetracycline in 10 ml. aqueous solution injected directly into a vein over a five-minute period. Blood specimens were collected at ½-hour, 1-hour, 3-hour, 6-hour, 12-hour, and 24-hour intervals following administration.

A specimen of blood was obtained from the patients just prior to the giving of the antibiotic on each occasion. This was run as a control sample. New vacuum blood-collecting tubes were used to collect the blood specimens to avoid the possibility of antibiotic-contaminated syringes and needles. The blood was obtained from superficial veins of the lower arms, with the usual precautions for sterility. These specimens were carried immediately to the laboratory and the serum was separated after the clot retracted. The serum was then frozen and the tests performed the following morning.

From the Department of Medicine, Baylor University College of Medicine and the Ben Taub Infectious Disease Laboratory, Jefferson Davis Hospital, Houston, Tex.

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The concentration of the drug in the serum was determined by a modification of the standard twofold serial dilution method of Rammelkamp.[2] The exact technique used was that described by English and associates[3] except that ascitic fluid was substituted for pooled serum. The test was carried out as follows: The medium used was brain heart infusion broth (Difco) to which ascitic fluid was added to a final concentration of 10 per cent. The test organism was a standard culture of *Streptococcus pyogenes* (ATCC8668), incubated for 18 hours and diluted to 1×10^{-3} in brain heart infusion broth containing 10 per cent ascitic fluid. The standard used in the studies was a concentration of tetracycline of 10 μg per milliliter. In the setting up of the standard, 11 tubes were used. To each tube, except No. 1 was added 0.5 ml. of the medium. The antibiotic standard was serially diluted from 5 μg. per milliliter in Tube 1 to 0.009 μg per milliliter in Tube 10. Tube 11 was used as a growth control and contained no antibiotic. To Tubes 1 and 2, 0.5 ml. of the tetracycline solution was added. The contents of Tube 2 were mixed, and 0.5 ml. was transferred to Tube 3 and mixed. Dilutions were carried out through Tube 10, and 0.5 ml. was discarded from this tube. Pipettes were changed between tests but not between tubes of the same test. To each tube was then added 0.5 ml. of the inoculum containing the Streptococcus, and the tubes were incubated at 37° C. for 18 to 20 hours.

Similarly, 11 tubes were set up for each serum specimen to be tested. To Tubes 2 through 11 was added 0.5 ml. of the medium. To Tubes 1 and 2 was added 0.5 ml. of the unknown serum. The contents of Tube 2 were mixed and 0.5 ml. was transferred to Tube 3. Dilutions were carried out through Tube 10, and 0.5 ml. was discarded from that tube. To each tube was added 0.5 ml. of the inoculum, and this was incubated in a manner similar to the standard.

The concentrations of the drug in the serum specimen were determined by comparing the tubes in which gross turbidity was inhibited with those of the standard. The product of the lowest concentration of the standard-inhibiting turbidity and the greatest dilution of the serum-inhibiting turbidity was equivalent to the concentration of the drug in the serum. Standards were determined with each group of specimens daily. It was found that a drug concentration of 0.039 μg per milliliter inhibited gross turbidity in most of the tests.

During the first stages of this study it was noted that the patients did not tolerate the large doses of the drug very well when given by intramuscular injection. Therefore an additional group of patients receiving the antibiotic for therapeutic reasons was selected, and their tolerance to intramuscular injections was observed. These patients were given either 100 mg. or 250 mg. injections at eight-hour intervals in the buttocks, alternating sides. These results were evaluated as to pain described by the patient and observed tissue reaction. These were recorded for pain as: negative—no pain; one plus—mild pain on questioning; two plus—moderate pain described; three plus—patient complained of severe pain; and four plus—patient refused injections because of pain. Observed tissue reaction was recorded as: negative—absent reaction; one plus—tenderness present; two plus—tenderness and induration present; three plus—swelling and redness present; and four plus—necrosis or abscess present.

RESULTS

In the group of patients receiving tetracycline as a single intramuscular injection, peak levels were reached between the first and fourth hour. The levels began to drop after that period but remained relatively high until after eight hours. The average levels for the various doses at the different time intervals are shown in Tables I, II, III, IV, and Fig. 1. Those patients receiving 50 mg. had serum levels ranging between 0.00 and 0.16 μg/ml. at one hour with an average of 0.12 μg/ml. and between 0.08 and 0.16 μg/ml. at eight hours, with an average of 0.08 μg/ml. Those receiving 100 mg. had serum levels ranging between 0.16 and 0.31 μg/ml. at one hour, with an average of 0.26 μg/ml.

and between 0.08 and 0.16 μg/ml. at eight hours, with an average of 0.15 μg/ml. Those receiving 250 mg. had serum levels ranging between 0.62 and 1.24 μg/ml. at one hour, with an average of 0.93 μg/ml. and between 0.31 and 0.62 μg/ml. at eight hours, with an average of 0.45 μg/ml. Those receiving 500 mg. had serum levels ranging between 2.48 and 4.96 μg/ml. at one hour with an average of 3.1 μg/ml. and between 0.62 and 1.24 μg/ml. at eight hours, with an average of 1.74 μg/ml. and 1 patient had a level of 4.96 μg/ml. Detectable serum levels were still present at twenty-four hours after injection, and in those patients receiving 500 mg. injections, the levels remained relatively high at twenty-four hours. In those patients receiving 50 mg. injections, the serum levels were quite low.

TABLE I. THE CONCENTRATION OF TETRACYCLINE (μG/ML.) IN THE SERUM FOLLOWING THE SINGLE INTRAMUSCULAR INJECTION OF 50 MG.

PATIENTS	HOURS AFTER ADMINISTRATION				
	1	4	8	12	24
M. Y.	0.16	0.16	0.08	0.08	0.00
M. M.	0.16	0.16	0.08	0.08	0.08
J. S.	0.00	0.00	0.00	0.00	0.00
J. A.	0.16	0.16	0.16	0.16	0.16

TABLE II. THE CONCENTRATION OF TETRACYCLINE (μG/ML.) IN THE SERUM FOLLOWING THE SINGLE INTRAMUSCULAR INJECTION OF 100 MG.

PATIENTS	HOURS AFTER ADMINISTRATION				
	1	4	8	12	24
R. G.	0.31	0.16	0.16	0.08	0.08
W. J.	0.16	0.31	0.16	0.08	0.08
P. A.	0.31	0.16	0.16	0.16	0.08
J. B.	0.31	0.08	0.08	0.00	0.00
F. J.	0.16	0.31	0.16	0.16	0.16
S. J.	0.31	0.16	0.16	0.08	0.08

TABLE III. THE CONCENTRATION OF TETRACYCLINE (μG/ML.) IN THE SERUM FOLLOWING THE SINGLE INTRAMUSCULAR INJECTION OF 250 MG.

PATIENTS	HOURS AFTER ADMINISTRATION				
	1	4	8	12	24
C. M.	0.62	0.31	0.31	0.31	0.16
J. A.	0.62	0.62	0.31	0.16	0.16
I. H.	0.62	0.31	0.31	0.16	0.08
E. R.	0.62	0.31	0.31	0.16	0.16
A. G.	0.62	0.31	0.16	0.16	0.08
C. I.	2.48	2.48	1.24	1.24	0.62
E. R.	1.24	0.62	0.62	0.62	0.31
S. W.	0.62	0.31	0.31	0.31	0.16

TABLE IV. THE CONCENTRATION OF TETRACYCLINE (μG/ML.) IN THE SERUM FOLLOWING THE SINGLE INTRAMUSCULAR INJECTION OF 500 MG.

PATIENTS	HOURS AFTER ADMINISTRATION				
	1	4	8	12	24
O. S.	2.48	1.24	0.62	0.62	0.31
C. B.	2.48	1.24	1.24	1.24	0.62
W. B.	4.96	4.96	4.96	2.48	1.24
G. S.	2.48	1.24	1.24	1.24	0.62
F. P.		1.24	0.62	0.62	0.31

TABLE V. THE CONCENTRATION OF TETRACYCLINE (μG/ML.) IN THE SERUM FOLLOWING THE INTRAMUSCULAR INJECTION OF 50 MG. EVERY EIGHT HOURS

PATIENTS	DAYS OF ADMINISTRATION				
	1ST	2ND	3RD	4TH	5TH
V. H.	0.31	0.31	0.31	0.31	0.31
B. M.	0.31	0.31	0.31	0.31	0.31

TABLE VI. THE CONCENTRATION OF TETRACYCLINE (μG/ML.) IN THE SERUM FOLLOWING THE INTRAMUSCULAR INJECTION OF 100 MG. EVERY EIGHT HOURS

PATIENTS	DAYS OF ADMINISTRATION				
	1ST	2ND	3RD	4TH	5TH
F. J.	0.31	0.62	0.62	0.62	0.62
W. R.	0.62	0.62	0.62	0.62	0.62
H. R.	0.31	0.62	0.62	0.31	0.31
R. T.	0.31	0.31	0.62	0.62	0.62
G. C.	0.62	0.62	0.62	0.62	0.62
V. C.	0.31	0.62	0.31	0.62	1.24
L. H.	0.62	0.62	0.62	0.31	1.24
M. G.	0.31	0.31	0.62		
R. B.	0.62	0.62	0.62	0.31	0.62

TABLE VII. THE CONCENTRATION OF TETRACYCLINE (μG/ML.) IN THE SERUM FOLLOWING THE INTRAMUSCULAR INJECTION OF 250 MG. EVERY EIGHT HOURS

PATIENTS	DAYS OF ADMINISTRATION				
	1ST	2ND	3RD	4TH	5TH
J. L.	0.62	0.62	1.24		
C. L.	1.24	2.48	1.24	1.24	1.24
N. S.	0.16	0.62	0.31	0.62	
K. G.	1.24	1.24	0.62	1.24	1.24
L. L.	0.31	0.62	1.24	1.24	1.24

TABLE VIII. THE CONCENTRATION OF TETRACYCLINE (μG/ML.) IN THE SERUM FOLLOWING THE SINGLE INTRAVENOUS INJECTION OF 100 MG.

PATIENTS	HOURS AFTER ADMINISTRATION					
	½	1	3	6	12	24
M. W.	0.62	1.24	0.62	0.31	0.00	0.00
L. J.	0.62	0.62	0.31	0.16	0.08	0.08

TABLE IX. THE CONCENTRATION OF TETRACYCLINE (μG/ML.) IN THE SERUM FOLLOWING THE SINGLE INTRAVENOUS INJECTION OF 250 MG.

PATIENTS	HOURS AFTER ADMINISTRATION					
	½	1	3	6	12	24
N. V.	2.48	1.24	1.24	1.24	0.62	0.31
L. R.	4.96	2.48	1.24	0.62	0.31	0.16
M. J.	2.48	1.24	1.24	1.24	0.62	0.31

TABLE X. THE CONCENTRATION OF TETRACYCLINE (μG/ML.) IN THE SERUM FOLLOWING THE SINGLE INTRAVENOUS INJECTION OF 500 MG.

PATIENTS	HOURS AFTER ADMINISTRATION					
	½	1	3	6	12	24
H. C.	19.84	9.92	4.96	4.96	0.31	
J. L.	9.92	9.92	4.96	4.96	1.24	0.31
D. Mc.	9.92	4.96	4.96	2.48	1.24	0.62

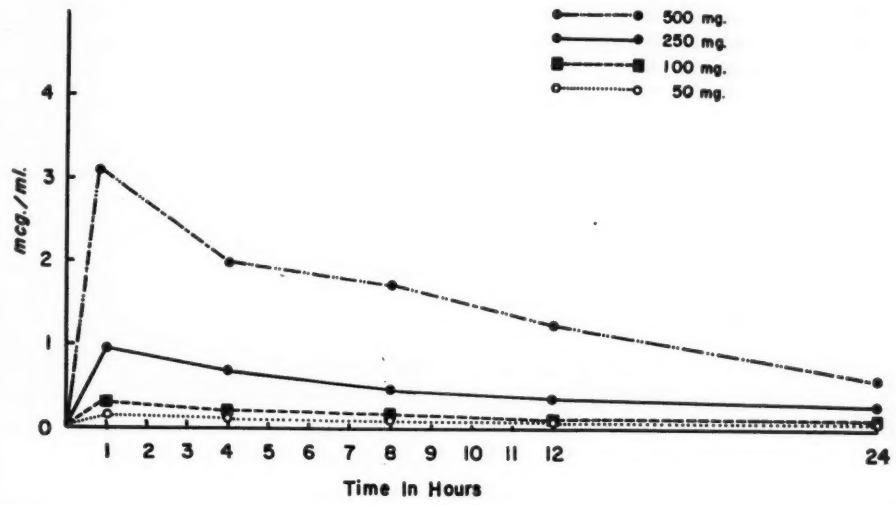

Fig. 1.—The average concentrations of tetracycline in the serum following single intramuscular injections.

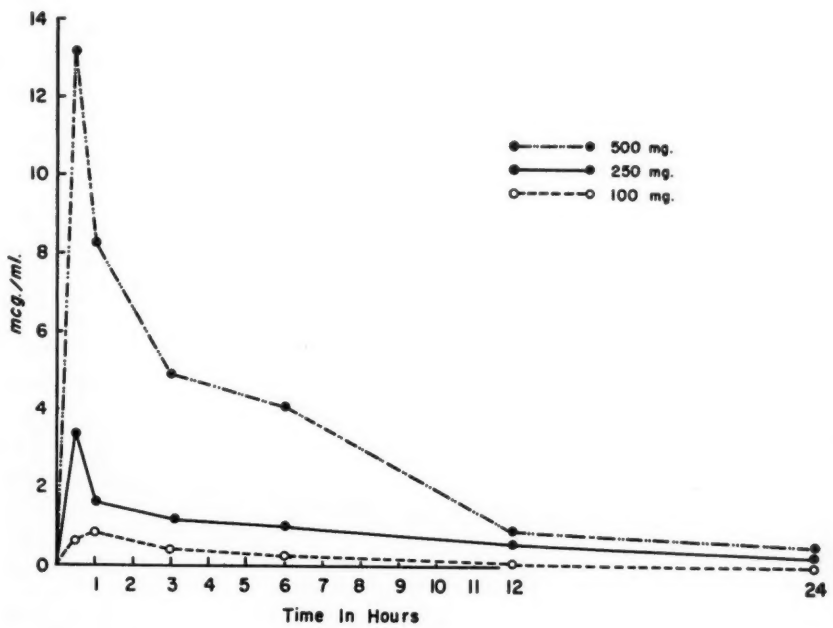

Fig. 2.—The average serum concentrations of tetracycline following single intravenous injections.

In the group of patients receiving multiple injections over a five-day period, an attempt was made to evaluate the accumulative effect of continued therapy. Tables V, VI, and VII show the average serum levels each day of therapy for the various dose schedules. Those patients receiving 50 mg. of the drug showed no evidence of accumulation, reaching maximum level of 0.31 μg/ml. Those receiving 100 mg. had an average level ranging between 0.31 and 1.24 μg/ml. on the fifth day, and those receiving 250 mg. had an average level of 1.24 μg/ml. on the fifth day.

Six of the patients in this series had marked impairment of liver function. These were: Table I—J. S.; Table III—C. M., J. A., E. R.; Table IV—O. S.; and Table VI—F. J. Five of the patients had evidence of impaired renal function, as evidenced by elevated blood urea nitrogen levels. These were: Table II—W. J., F. J.; Table III—C. I., S. W.; Table IV—C. B. These patients had tetracycline serum levels comparable with the other patients in their respective groups at the various time intervals. It is felt that in these patients neither hepatic nor renal impairment altered the metabolism and excretion of tetracycline.

In Tables VIII, IX, X, and Fig. 2, the results of single intravenous injections of the drug are presented. The highest levels were obtained within one hour following the administration of the drug, ranging between 0.62 and 1.24 μg/ml. in those receiving 100 mg. with an average of 0.93 μg/ml., and between 2.48 and 4.96 μg/ml. in those receiving 250 mg. with an average of 3.28 μg/ml. At six hours following administration of the drug these had dropped to levels ranging between 0.16 and 0.31 μg/ml. in those receiving 100 mg., with an average of 0.16 and 0.31 μg/ml., in those receiving 100 mg., with an average of 0.24 μg/ml., between 0.62 and 1.24 μg/ml., in those receiving 250 mg., with an average of 1.03 μg/ml., and between 2.48 and 4.96 μg/ml. in those receiving 500 mg., with an average of 4.13 μg/ml.

Those patients receiving 100 mg. injections every eight hours had only mild local reactions. Only 1 patient complained of severe pain. Two patients complained of moderate pain and 3 admitted slight pain on questioning, whereas 4 patients denied any pain. Of these patients, 2 had tenderness with induration in the injection sites, 1 had tenderness only, and 7 had no local reactions.

Of those patients receiving 250 mg. of the drug every eight hours, 2 refused further injections after four days because of pain, 5 complained of severe pain, 4 had moderate pain, and 2 complained of only mild pain. Four of these patients had swelling with redness and tenderness at the site of injection. Four had tenderness with induration of the site of injection, 1 had tenderness only, and 4 had no local reaction.

In general, it was found that patients tolerated 100 mg. doses very well; however, 250 mg. doses produced significant local reactions to cause marked discomfort in the patient.

CONCLUSIONS AND SUMMARY

1. The serum levels of tetracycline following the intramuscular administration of various dosages are presented. These are separated into 2 groups: one

in which single injections are evaluated and the other in which the accumulative effect of prolonged administration is evaluated. A comparison is made to a limited number of determinations following the intravenous administration of the drug.

2. After single intramuscular injections, sustained levels were produced for twelve hours, with a maximum ranging up to 0.16 μg/ml. following 50 mg. doses, 0.16 and 0.31 μg/ml. following 100 mg. doses, 0.62 and 1.24 μg/ml. following 250 mg. doses, and 2.48 and 4.96 μg/ml. following 500 mg. doses. These resulting blood levels are higher than those previously reported following the oral administration of equivalent quantities of the antibiotic, being approximately twice as high.

3. After multiple intramuscular injections at eight-hour intervals over a five-day period, the maximum serum level of tetracycline following 50 mg. injections was 0.31 μg/ml., while that following 100 mg. injections ranged between 0.31 and 1.24 μg/ml., and that following 250 mg. injections was 1.24 μg/ml. These results showed a tendency toward an accumulative effect in those patients receiving 250 mg. doses. The multiple injection of 250 mg. of the drug was accompanied by so much pain at the site of injection it was frequently difficult to persuade the patients to continue on the drug.

4. Single intravenous injections of the drug produced a quite high blood level which quickly dropped in a period of six hours.

5. Blood levels of tetracycline were not altered by either marked hepatic or marked renal impairment.

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THE METABOLISM OF WATER AND ELECTROLYTES IN PATIENTS WITH CIRRHOSIS OF THE LIVER

PETER J. TALSO, M.D.,* IRVIN H. STRUB, M.D.,** AND JOSEPH B. KIRSNER, M.D.
CHICAGO, ILL.

INTRODUCTION

THE pathogenesis of the ascites and edema in cirrhosis of the liver remains incompletely understood. Recent studies emphasize the multiple factors under scrutiny as possible mechanisms in the abnormal accumulations of fluid in this disease.[1-18] The retention of fluid in cirrhosis is associated with retention of the sodium ion, presumably resulting from increased renal tubular reabsorption of this cation. Most recently this abnormality of sodium excretion has been attributed to the effects of aldosterone.[19]

The present study was undertaken to further elucidate the nature of the defect in electrolyte and water metabolism in cirrhosis of the liver, by determining the distribution of electrolytes and water in samples of skeletal muscle obtained from the deltoid and gastrocnemius, and by correlating these observations with data derived from metabolic balance studies performed during the removal of edema fluid by medical means.

METHODS AND MATERIALS

The subjects were six male patients with cirrhosis of the liver complicated by ascites and edema and one healthy male student with normal hepatic function. In the patients studied, the diagnosis of cirrhosis of the liver was established by history, physical examination, laboratory evidence indicative of hepatocellular disease, and/or by needle biopsy of the liver. All patients were maintained in the metabolic section of the Albert Merritt Billings Hospital throughout the study. Initial muscle biopsies were obtained in all six cirrhotic patients; however, metabolic balance studies were carried out in only four. One of the patients was considered undesirable for the balance studies because of chronic glomerulonephritis, and an episode of massive hematemesis from a ruptured esophageal varix precluded balance studies in the second.

Diets.—Following admission to the metabolic ward, the patients were permitted to select diets to conform with their customary food habits. The diets then were adjusted to provide the desired amounts of carbohydrates and proteins and to maintain the salt intake at approximately 1.5 Gm. per day. More fat was allowed than usual in the interest of palatability. During the first two phases of the study a weighed amount of sodium chloride was added to raise the salt intake to approximately 3.0 Gm. With the onset of the metabolic

From the Departments of Medicine of the University of Chicago and the Stritch School of Medicine of Loyola University, Chicago, Ill.

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*Present address: The Stritch School of Medicine of Loyola University.

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studies, the diets thus determined were kept scrupulously constant. The only variation in the diets throughout the period of study was the change in the salt intake from 3.0 to 1.5 Gm. of salt. The carbohydrate, protein, fat, and total caloric intake of the individual diets are shown in Table I.

TABLE I. COMPOSITION OF DIETS

PATIENT	CARBOHYDRATE (GM.)	PROTEIN (GM.)	FAT (GM.)	TOTAL CALORIES
D. N.	313	84	88	2,380
A. O.	258	94	113	2,425
J. O.	197	83	98	1,957
F. B.	288	96	88	2,328
I. D.	265	61	134	2,510

After the obtaining of the initial muscle biopsies in each patient, balance studies were performed under the following conditions: (a) control period with the diet containing 3.0 Gm. of sodium chloride daily; (b) the same diet plus Carbo-Resin,* 45 Gm. daily; (c) decrease in the daily salt intake to 1.5 Gm., the Carbo-Resin continued; and (d) the identical program plus a mercurial diuretic, Dicurin,* 2 ml. subcutaneously every second day. With the completion of the metabolic studies a final set of skeletal muscle biopsies were obtained in the cirrhotic patients.

ANALYTICAL METHODS

Diets.—An aliquot of one day's diet for each week of the study was homogenized in a Waring blendor, quantitatively transferred to a 2,000 ml. volumetric flask, and diluted to volume with distilled water (a drop of caprylic alcohol was added to prevent foaming.) Twenty-five milliliter aliquots of this suspension were transferred to 50 ml. tared silica beakers, with use of a calibrated siliconized 25 ml. pipette. The samples were dried to constant weight in an oven at 95° C. After cooling they were weighed to assure consistency in the quantities of quadruplicate aliquots. The food samples then were treated with 5 ml. of 4 N sulfuric acid and heated in the drying oven until only a black charred residue remained; this preliminary treatment avoided spattering and loss of material. The samples then were placed in a muffle furnace at 600° C. for 36 hours. After cooling the dried residue was redissolved in 1 N hydrochloric acid, quantitatively transferred to volumetric flasks, and diluted to volume; aliquots of this solution were utilized for sodium and potassium determinations with use of the Weichselbaum-Varney flame spectrophotometer. The chloride content was measured on 25 ml. aliquots of the diet suspension with use of the Wilson and Ball[20] modification of the Van Slyke method.[21] Dietary nitrogen was determined on similar aliquots by the micro-Kjeldahl technique.[22]

Feces.—Stools were collected at the end of three- and four-day alternate periods, marked with carmine. The entire three- or four-day stool collection was transferred quantitatively to a Waring blendor, homogenized, and diluted to a volume of 4,000 ml. Aliquots of this suspension then were handled and analyzed by methods identical to those used for diet analyses.

Urine.—Twenty-four-hour collections of urine were made daily, with use of thymol as a preservative. These were analyzed for: sodium, potassium, chloride, and nitrogen, by methods noted previously; ammonia was determined by the Conway microdiffusion technique[23]; urinary pH with a Beckman pH meter; and creatinine by the Hoffman modification[24] of the Folin-Wu method.[25]

Blood and Serum.—Samples of venous blood were drawn at intervals of three or four days in oiled syringes utilizing minimal stasis. An aliquot of the whole blood was defibrinated immediately by "whipping" with several wooden applicators. Aliquots of the defibrinated blood were utilized for the determination of the hematocrit, whole blood water, chloride, sodium, and potassium. The remainder of the blood was allowed to clot under oil and after

*Kindly supplied by Eli Lilly & Company.
Composition of resin: anion exchange resin 12 per cent, potassium salt of carboxylic acid resin 29 per cent, carboxylic acid resin (hydrogen cycle) 59 per cent.

centrifugation, the serum was removed by anaerobic technique, and aliquots were utilized for the determination of bicarbonate by the Van Slyke method[26]; pH, with the Beckman pH meter; chloride, by the Wilson and Ball modification of the Van Slyke method; and sodium and potassium were determined with the Weichselbaum-Varney flame spectrophotometer.

Skeletal Muscle.—The muscle biopsies were secured in the operating room under regional nerve block anesthesia and with standard aseptic technique.* The specimens, weighing 4 to 10 grams, were placed in tightly sealed jars and transported promptly to the laboratory. Analyses were begun within five minutes after removal of the biopsies from the patients. The visible fat and connective tissues were dissected away, and the specimen was blotted lightly with filter paper to remove excess blood and then minced thoroughly with a pair of scissors. Duplicate samples of muscle were weighed, placed in a drying oven at 95° C., dried for 48 hours, and then they were transferred to a desiccator, allowed to reach room temperature, and reweighed. The difference between the initial weight and the dry weight constituted the water content of the sample. The dried tissues then were extracted with diethyl ether until the extractant was colorless. The samples were dried once again and reweighed; the difference between the weight of the dry tissue and the weight after ether extraction constituted the content of neutral fat. Nitrogen, chloride, sodium, and potassium were determined on weighed aliquots, by methods described previously.[27]

CALCULATIONS

Phase-mass data for specimens of skeletal muscle were calculated by the method of Hastings and Eichelberger.[28] The metabolic balance data were analyzed by the methods of Reifenstein and Albright.[29]

RESULTS

Clinical.—All subjects, including the normal control, lost weight during the period of observation. Two patients experienced weight loss promptly after administration of the exchange resin and continued to do so throughout subsequent periods; the total losses of weight were 17.9 kg. and 16.6 kg., respectively. In one subject, weight loss did not commence until the dietary salt intake had been reduced to 1.5 Gm. daily; the total weight loss was 15.8 kg. The fourth patient experienced only a moderate weight loss (7.0 kg.), which progressed at an equal rate in all phases of the study. In the normal control subject, the weight loss during the study was 5.2 kg.; however, his initial weight was 106.8 kg. and he received a diet deficient in protein and calories as compared with his customary intake.

Metabolic Data.—Illustrative analytical data on one of the patients and on the normal control subject are presented in Tables II and III and in Figs. 1 and 2. Analytical data and figures for the remaining three patients are not included because of the massive nature of the tables.

Serum: In three of the four patients with cirrhosis, hyponatremia (sodium less than 135 meq./L.) developed toward the conclusion of the study, however, no clinical symptoms were apparent. The serum sodium did not change significantly in the control subject.

Hypokalemia (potassium less than 3.5 meq./L.) developed in all four patients at some time during the period of observation. In three cases the serum potassium decreased below 3 meq./L. There were no symptoms referable to the hypokalemia in three patients; one individual manifested weakness and lethargy after the final muscle biopsy; these symptoms occurred at a time when

*The anesthesia was administered by Dr. Donald Benson, Department of Anesthesiology; the biopsies were performed by Dr. Ralph Carlson, Department of Surgery, the University of Chicago; we are greatly indebted to them for their skillful assistance.

he also had hyponatremia and subsided following the administration of broth and fruit juices containing potassium salts. The metabolic data were not altered since this incident occurred after the balance studies had been completed. No significant change in serum potassium concentrations occurred in the control subject as a result of resin therapy.

Prior to therapy the serum chloride concentrations were normal and then rose slightly in three of the four patients after administration of the exchange resin. The serum chlorides decreased slightly in all cases after injection of the mercurial diuretic. The serum bicarbonate and serum pH tended to decrease slightly during resin therapy and then increased during the administration of the mercurial diuretic. The total proteins, the albumin globulin ratio, and the blood urea nitrogen did not change significantly during the period of observation.

Balance Data.—

Sodium: The sodium balance was positive during the initial phase of observation in the four patients and was reflected by a low urinary and fecal excretion of sodium. Administration of the exchange resin invariably increased the excretion of sodium in the feces while the urinary content decreased simultaneously. During the final period of observation the urinary output of sodium increased many fold following each injection of the mercurial diuretic. The cumulative negative sodium balances during therapy in subjects D. N., A. O., J. O., and F. B. during the entire period of observation were: 746.4,-429.3,-1,791.2 and 1,359.4 meq., respectively. In the control subject the negative sodium balance was –126.4 meq.

Potassium: In all four patients the potassium balance was negative initially. This trend toward a negative potassium balance became less pronounced during the administration of the resin. The fecal potassium content increased greatly, in part because the potassium salt of the carboxylic acid resin constituted 29 per cent of the resin; however, the resin removed an additional amount of potassium from the gastrointestinal contents and, concomitantly, the potassium output in the urine decreased. The net result was a negative potassium balance in three patients. In the fourth patient the potassium balance, initially variable, became positive toward the close of the study. In the normal subject the potassium balance was consistently slightly negative.

Chloride: The chloride balance was positive during the initial period of observation in all patients as well as in the control. During the administration of the resin, the chloride balance became negative, reflecting the increased urinary excretion of this ion. Fecal chloride was not influenced significantly by the resin, probably because anions other than chloride, such as $Po_4 \equiv$, $HPO_4 =$ and $SO_4 =$, possess a greater affinity for the anion exchanger incorporated into the resin mixture. During the final period of observation, the chloride balance became more negative as a result of the chloruretic effect of the mercurial diuretic.

Urinary ammonia: The urinary excretion of ammonia increased three to sixfold over control values during the administration of the resin, reflecting the increased synthesis of ammonia by the renal tubules in response to the hyperchloremic acidosis induced by the resin.

TABLE II. CIRRHOSIS OF LIVER WITH

DAYS	REGIME (NO.)	BODY WGT. (KG.)	TOTAL INTAKE 1 NA+	1 K+	1 CL−	2 N	URINE 3 (ML. AVERAGE VOL.)	1 NA+	1 K+	1 CL−	4 NH_4^+	2 N	5 PH
1- 3	1	90.7	148.2	236.1	136.2	40.5	2.234	1.5	180.3	40.0	55.0	26.8	
4- 7	1	90.6	197.6	314.8	181.6	54.0	2,282	1.3	230.6	57.3	54.6	38.0	6.3
8-10	1	91.8	148.2	236.1	136.2	40.5	2,542	2.4	173.1	52.1	52.1	27.2	6.2
11-14	1	91.9	197.6	314.8	181.6	54.0	3,052	3.9	217.0	54.5	56.8	35.9	6.4
15-17	2	93.1	146.1	435.0	123.3	40.8	3,905	3.3	113.1	60.3	193.5	27.0	8.7
18-21	2	92.4	194.8	580.0	164.4	54.4	3,789	4.6	62.4	154.3	235.5	31.3	8.5
22-24	2	92.5	146.1	435.0	123.3	40.8	3,571	4.0	42.6	157.9	239.6	23.0	8.0
25-28	2	92.9	194.8	580.0	164.4	54.4	3,804	6.1	48.6	179.8	245.0	35.1	8.5
29-31	2	93.0	146.1	435.0	123.3	40.8	3,679	2.3	50.0	145.8	251.7	25.6	8.8
32-35	2	92.6	194.6	580.0	164.4	54.4	3,314	3.5	55.8	187.7	261.6	31.3	8.8
36-38	2	93.9	146.1	435.0	123.3	40.8	3,614	5.4	57.6	168.9	252.6	26.6	8.7
39-42	3	93.1	104.8	582.0	120.8	53.6	3,889	8.6	87.6	200.7	249.2	35.1	8.6
43-45	3	92.0	78.6	436.5	90.6	40.2	3,946	7.5	67.4	141.4	247.8	25.9	8.6
46-49	3	91.2	104.8	582.0	120.8	53.6	3,876	11.0	71.4	184.1	183.0	36.6	9.2
50-52	3	89.5	78.6	436.5	90.6	40.2	3,645	7.5	55.7	136.0	234.2	27.5	9.3
53-56	4	89.7	104.8	582.0	120.8	53.6	4,467	329.8	194.1	611.2	222.0	38.8	8.2
57-59	4	86.4	78.6	436.5	90.6	40.2	4,353	242.2	143.3	396.8	263.0	30.9	8.5
60-63	4	84.9	104.8	582.0	120.8	53.6	3,859	140.8	137.4	289.8	317.7	41.1	8.8
64-66	4	82.0	78.6	436.5	90.6	40.2	3 910	70.9	78.1	133.4	193.0	31.9	8.0
67-70	4	80.7	104.8	582.0	120.8	53.6	3,882	161.9	134.3	303.2	306.2	42.7	8.7

Regime 1: 3 Gm. NaCl diet.
Regime 2: 3 Gm. NaCl diet + 45 Gm. resin.
Regime 3: 1.5 Gm. NaCl diet + 45 Gm. resin.
Regime 4: 1.5 Gm. NaCl diet + 45 Gm. resin + 2 c.c. Hg diuretic q.o.d.

TABLE III. NORMAL

DAYS	REGIME (NO.)	BODY WGT. (KG.)	TOTAL INTAKE 1 NA+	1 K+	1 CL−	2 N	URINE 3 ML. AVERAGE VOL.	1 NA+	1 K+	1 CL−	4 NH_4	2 N	5 PH
1-3	1	106.5	135.6	243.9	125.7	30.3	784	63.7	161.1	85.2	75.4	43.9	5.5
4-7	2	105.4	188.8	588.0	168.0	39.6	1,449	32.0	272.6	162.0	101.8	60.5	5.7
8-10	2	104.1	141.6	441.0	126.0	29.7	1,428	4.1	134.2	140.4	126.4	44.1	5.9
11-14	3	103.3	99.2	605.6	112.0	38.8	1,986	2.7	131.4	183.8	141.8	69.6	6.1
15-17	3	103.1	74.4	454.2	82.0	29.1	2,086	6.0	74.4	112.8	150.7	59.4	6.1
18	4	101.6	24.8	151.4	28.0	9.7	2,077	23.6	35.7	61.1	135.4	15.9	6.0

Regime 1: 3 Gm. NaCl diet.
Regime 2: 3 Gm. NaCl diet + 45 Gm. resin.
Regime 3: 1.5 Gm. NaCl diet + 45 Gm. resin.
Regime 4: 1.5 Gm. NaCl diet + 45 Gm. resin + 2 c.c. Hg diuretic q.o.d.

Acid-base metabolism: As noted above, the serum bicarbonate and serum pH decreased slightly during the initial phase of resin therapy, reflecting the mild hyperchloremia induced by the resin. The prompt adjustment of the renal mechanism to the increased anion load was manifested by the prompt decrease in pH of the urine in three cases and by the very prompt increase in urinary ammonia in all patients. In one patient, D. N., the pH of the urine rose following the administration of the resin and remained so throughout the study. The explanation for this unusual phenomenon could not be ascertained; unfortunately, the urinary content of total base, bicarbonate, and other buffer substances was not determined. Administration of the mercurial diuretic led to a slight increase in urine pH in three patients, reflecting the increased excretion

ASCITES AND EDEMA—BALANCE DATA D. N. #545,606

STOOL				SERUM						WHOLE BLOOD				
1	1	1	2	6	7	8	8	8	9	10	9	8	8	8
NA^+	K^+	CL^-	N	PH	HCO_3^-	NA^+	K^+	CL^-	H_2O	HCT	H_2O	NA^+	K^+	CL^-
1.8	64.2	2.1	5.7	7.4	27.6	132	5.7	99.0	939.2	40.3	844.4	81.0	41.5	80.6
2.0	109.2	4.0	9.6	7.5	31.5	135	4.2	99.2	926.3	37.0	857.1	84.0		
0.6	45.0	1.5	4.5	7.4	30.5	137	4.0	100.0	934.5	36.3	852.8	86.0	38.0	84.0
5.6	81.6	2.4	6.8	7.5	29.1	141	4.2	99.3	941.3	34.4	863.7	88.0	39.5	83.2
62.4	415.2	4.5	7.5	7.4	30.9	140	3.5	103.6	936.4	36.4	866.3	90.5	38.5	84.2
126.0	552.0	25.2	11.2	7.4	28.6	138	3.3	101.7	932.5	36.4	858.2	88.0	40.5	85.8
109.2	412.8	14.1	8.7	7.4	29.8	138	3.4	101.8	929.8	35.9	852.6	92.0	34.5	86.0
133.4	488.0	16.0	10.0	7.4	27.2	139	3.3	102.5	930.4	33.9	853.3	100.0	36.0	87.4
105.9	398.1	13.8	7.5	7.4	28.4	139	3.6	100.6	930.2	30.6	853.8	90.5	37.5	
178.4	516.0	4.4	10.8	7.4	28.8	139	3.3	100.6	919.0	34.5	871.0	92.0	33.5	86.4
137.4	338.4	2.1	8.1	7.4	29.6	136	3.3	99.2	922.7	32.7	855.2	90.5	41.5	
142.0	347.6	2.8	8.4	7.5	31.4	134	3.2	99.2	924.9	35.5	854.1	93.0	34.2	84.8
154.8	390.0	11.1	8.1	7.4	29.0	140	3.2	100.4	925.7	32.9	845.2	102.5	37.2	
222.0	536.0	26.4	6.4	7.6	28.2	144	3.5	103.1	930.3	33.0	858.0	101.0	35.0	86.3
138.3	400.5	2.7	9.0	7.4	30.3	141	3.4	99.4	927.9	32.7	850.4	84.5	36.0	87.9
180.0	423.2	2.4	10.8	7.4	30.2	140	3.4	98.4	926.1	35.0	860.9	93.5	36.5	83.6
161.4	336.6	2.7	8.1	7.5	32.0	137	2.9	95.2	924.3	32.3	851.6	88.0	37.2	79.8
179.2	447.2	5.2	11.6	7.6	33.6	145	3.2	94.8	920.8	34.0	837.5	99.5	35.8	79.3
130.5	384.3	10.8	9.0	7.7	32.5	144	2.8	95.2	927.4	33.7	851.1	99.0	37.0	78.9
187.2	458.0	6.4	11.2	7.5	31.9	148	2.9	95.4	930.9	33.2	862.1	98.0	35.5	78.2

1. meq. per experimental period.
2. Gm. per experimental period.
3. Average volume per day for period.
4. Average meq. (NH_4) for experimental period.
5. Average urinary pH for experimental period.
6. Serum pH for first day of period.
7. mm./L. for first day of period.
8. meq./L. for first day of period.
9. Gm./L. for first day of period.
10. Per cent for first day of period.

INDIVIDUAL—BALANCE DATA I. D. #441,899

STOOL				SERUM						WHOLE BLOOD				
1	1	1	2	6	7	8	8	8	9	10	9	8	8	8
NA^+	K^+	CL^-	N	PH	HCO_3^-	NA^+	K^+	CL^-	H_2O	HCT	H_2O	NA^+	K^+	CL^-
7.8	55.5	2.7	3.0	7.4	18.9	136.0	4.8	100.4	921.4	50.3	823.9	79.5	52.2	79.3
108.0	425.6	4.8	5.2	7.3	24.6	140.5	5.0	102.4	927.9	47.6	812.0	81.5	51.8	80.2
144.0	411.9	3.3	3.6	7.4	26.4	139.5	4.9	104.4	917.9	53.9	809.5	79.5	55.0	80.4
80.0	502.8	5.6	7.2	7.3	25.6	141.5	4.4	104.3	923.4	51.5	813.6	79.5	52.2	80.6
26.4	459.9	2.7	3.3	7.4	27.8	138.5	4.3	80.6	918.2	53.3	815.0	77.5	52.0	78.4
21.4	130.7	1.1	1.3	7.3	26.0	141.5	4.1	99.9	915.5	53.9	808.8	78.5	55.0	78.4

1. meq. per experimental period.
2. Gm. per experimental period.
3. Average volume per day for period.
4. Average meq. (NH_4) for experimental period.
5. Average urinary pH for experimental period.
6. Serum pH for first day of period.
7. mm./L. for first day of period.
8. meq./L. for first day of period.
9. Gm./L. for first day of period.
10. Per cent for first day of period.

of body base; in one patient (D. N.) the urine was alkaline during resin administration and remained so following injection of the mercurial diuretic. The pH of the urine did not change significantly in the fourth patient (F. B.).

DERIVED METABOLISM DATA

Calculated values for the changes in body compartments during the various periods of study are illustrated in Figs. 1 and 2. Body protoplasm (muscle mass) increased throughout the period of observation in three of the patients

studied; however, one patient (A. O.) was in negative nitrogen balance throughout and in this case the protoplasmic mass decreased. The cause for this was not apparent from the available data. The nitrogen balance also was consistently negative in the control subject, and the protoplasmic mass diminished (Fig. 2). The volume of extracellular fluid, calculated from the change in chloride space, increased in three of the four patients during the first period of observation and then diminished progressively during the subsequent three periods. In three patients the changes in volume of extracellular fluid closely approximated the actual changes in body weight.

Changes in volume of intracellular water were calculated from the external potassium balance corrected for nitrogen by the methods of Reifenstein and Albright.[29] Although moderate losses of intracellular water occurred in all patients during the early phases of the study, the volume of this compartment increased slightly in three cases toward the conclusion of the observations. The volume of intracellular water did not vary significantly in the normal control subject.

The theoretical changes in body weight, calculated from the changes in corrected protoplasmic mass, in intracellular water, and in extracellular water as calculated from the chloride balance, closely corresponded to the changes in actual body weight (Figs. 1 and 2). In three cases the loss of weight represented the loss of extracellular fluid predominantly. In the fourth case the weight loss reflected the combined loss of protoplasmic mass, extracellular fluid and of intracellular water. In the control subject the weight loss coincided with the loss of actual muscle mass, as a result of the negative nitrogen balance (Fig. 2).

Hematocrit, Serum Water, Serum Density.—The hematocrits measured twice weekly varied but in no consistent pattern. The serum water and serum density did not change significantly throughout the course of the study in any of the four patients.

Tissue Studies: The analytical data on skeletal muscle are presented in Tables IV and VI; Table IV includes the six patients with massive ascites and peripheral edema from whom muscle biopsies were obtained initially. Table VI comprises the data obtained in the four patients in whom biopsies were performed at the completion of the study. The derived values in Tables V and VII and in Fig. 3 were calculated from the data in Tables IV and VI. Table VIII illustrates the method used in deriving the diagrams in Fig. 3.

Tissue water: The total water content of the samples of gastrocnemius and deltoid muscles before therapy was slightly higher than normal. The mean value for samples of deltoid muscle was 788.2 ± 9.0 Gm. of water per kilogram of fresh fat-free tissue and for gastrocnemius muscle 790.6 ± 18.6 Gm., as compared with the control figure, 776 ± 6.4 Gm, determined in a previous study.[27]

The mean value for intracellular water in the gastrocnemius muscle samples before therapy was 514.6 ± 19.6 Gm. per kilogram of fresh fat-free tissue and for the deltoid 561.4 ± 29.2 Gm., compared with a control value of 611.0 ± 30.8 Gm.

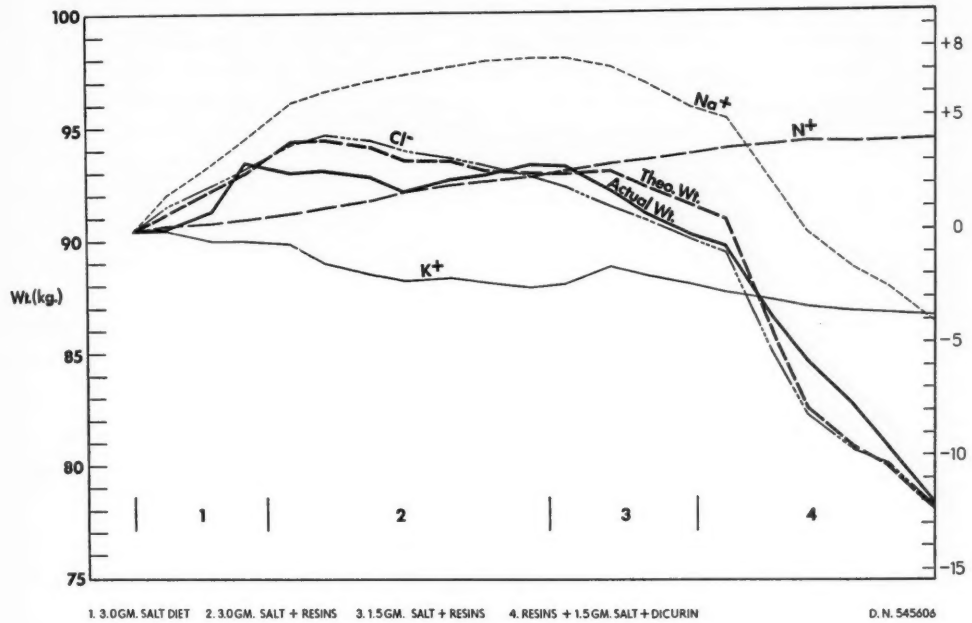

Fig. 1.

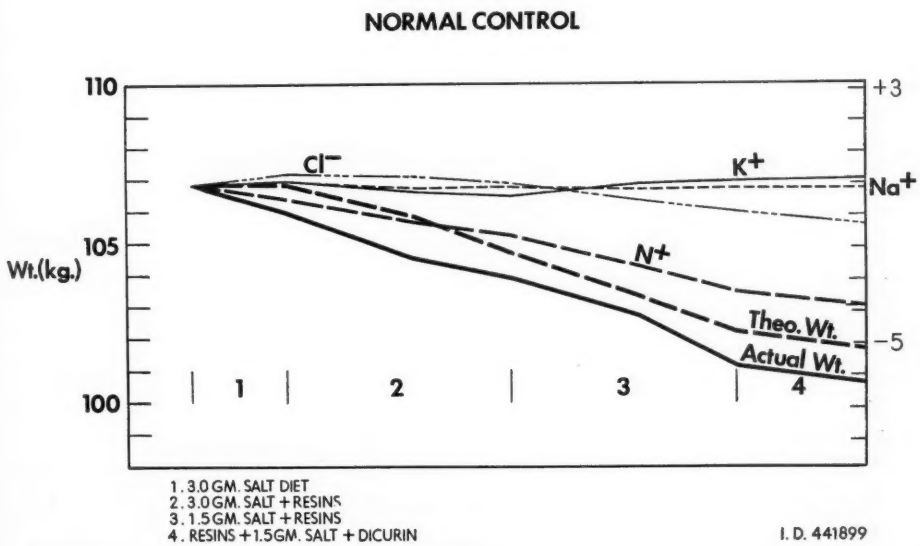

Fig. 2.

Figs. 1 and 2.—Changes in body compartments during therapy of cirrhosis of the liver. The line labeled Cl– represents the changes in extracellular fluid compartment calculated from the external chloride balance; Na+ represents the changes in extracellular fluid calculated from the external sodium balance; K+ represents the changes in the intracellular fluid compartment calculated from the external potassium balance; N+ represents the change in protoplasmic mass calculated from the nitrogen balance; the changes in theoretical weight were calculated by combining the values for changes in extracellular volume derived from chloride balance with the values for changes in intracellular water and protoplasmic mass.

The mean value for extracellular fluid in gastrocnemius muscle before therapy was 277.5 ± 35.0 Gm. per kilogram of fresh fat-free tissue and for deltoid muscle 226.8 ± 33.2 Gm. per kg., compared with a control value of 165.5 ± 33.3 Gm. per kg. The figures for extracellular water in the muscles of the cirrhotic group thus are distinctly higher than normal.

TABLE IV. ANALYSES OF SERUM AND MUSCLE TISSUES BEFORE TREATMENT

PATIENT	SITE		PH	CO_2 (MM./L)	H_2O* (GM.)	CL^-* (MEQ.)	NA^+* (MEQ.)	K^+* (MEQ.)	N* (GM.)	FAT (%)
		S†	7.4	30.5	937.2	99.0	139.5	4.2		
D. N.	D§	M‡			793.9	30.8	34.9	87.5	27.8	1.1
	G‖	M			818.2	37.3	47.2	81.0	23.0	2.8
		S	7.4	25.9	917.6	108.6	145.0	4.5		
A. O.	D	M			786.7	28.4	43.9	81.2	28.8	5.2
	G	M			792.1	31.6	41.2	84.3	28.3	5.7
		S	7.4	30.6	912.4	98.8	137.5	3.9		
J. O.	D	M			788.4	20.0	32.2	93.9	21.1	2.2
	G	M			775.8	32.5	47.3	83.0	26.6	1.3
		S	7.4	31.2	921.3	101.4	134.0	5.3		
F. B.	D	M			801.4	28.1	64.1	77.2	27.3	1.2
	G	M			806.0	34.4	70.0	72.3	26.9	2.3
		S			912.2	105.5	140.5	4.2		
E. R.	D	M			774.0	24.6	41.8	88.6	28.8	0.3
	G	M			775.7	31.0	48.6	82.6	24.5	0.2
		S	7.3	24.5	928.0	98.8	137.5	3.9		
T. C.	D	M			788.4	28.1	40.1	82.1	29.7	0.1
	G	M			775.8	28.7	51.7	86.8	24.9	0.1
Mean		S	7.4	28.5	921.5	102.0	139.0	4.3		
S.D.			0.4	3.1	9.7	4.1	3.7	0.5		
Mean		D			788.2	26.6	42.8	85.1	28.9	
S.D.					9.0	3.8	11.3	6.0	1.4	
Mean		G			790.6	32.6	51.0	81.7	25.7	
S.D.					18.2	3.0	9.9	5.0	1.9	
Controls										
Mean		S	7.41	26.4	910.8	100.0	136.5	4.34		
S.D.			0.04	2.8	4.6	3.0	2.5	0.31		
Mean		M			776.6	19.1	33.7	94.0	29.5	
S.D.					6.4	3.9	6.4	5.9	2.2	

*Expressed per kilogram of fresh fat-free tissue.
†S=Serum.
‡M=Muscle.
§D=Deltoid.
‖G=Gastrocnemius.
S.D.=Standard deviation.

The significance of the preceding results becomes most apparent when the values are referred to tissue solids. Thus, the total water content for the deltoid muscle, referred to tissue solids, is 834 Gm. and for the gastrocnemius muscle 862 Gm. When the figures for intracellular water as referred to tissue solids are compared in the various groups, the value for gastrocnemius (558.0 ± 40.6) is slightly lower than the control (611.0 ± 30.8) but does not differ significantly from the value for deltoid (593.8 ± 34.7). Similarly the percentage of water in the intracellular phase is slightly lower in the gastrocnemius muscle than

TABLE V. DERIVED ELECTROLYTE AND WATER DATA BEFORE TREATMENT

PATIENT	SITE	$(H_2O)F$ (GM./KG.) FAT-FREE	$(H_2O)C$ FRESH TISSUE	$(H_2O)C$ (GM./KG.) CELLS	(NA) (MEQ./KG.)	(K) INTRACELLULAR	(NA + K) H_2O	RATIO: (NA)M* (CL)M*	RATIO: (K)M* (N)M†
D. N.	D	275.8	518.4	718.6	0	164.9	164.9	1.1	3.2
	G	334.8	484.2	731.7	1.6	137.4	139.0	1.3	3.0
A. O.	D	236.3	550.4	723.0	11.1	145.8	157.0	1.5	2.8
	G	263.2	528.9	720.5	2.4	157.3	159.6	1.3	3.0
J. O.	D	179.0	605.6	739.3	7.1	153.6	160.8	1.6	3.0
	G	296.8	496.8	709.5	23.2	164.6	189.7	1.5	3.1
F. B.	D	229.9	571.5	744.3	43.1	133.5	176.6	1.1	2.8
	G	282.2	523.6	732.4	44.6	135.8	180.5	1.1	2.7
E. R.	D	202.0	572.0	718.6	16.0	153.7	169.7	1.7	3.1
	G	245.5	521.2	701.6	16.1	156.3	172.5	1.6	3.4
T. C.	D	237.9	550.5	724.6	2.6	147.2	149.8	1.4	2.8
	G	242.7	533.1	706.2	18.1	160.8	178.9	1.8	3.5
Mean	D	226.8	561.4	728.0	13.3	149.8	163.1	1.4	3.0
S.D.		±33.2	±29.2	±11.0	±15.7	±10.5	±9.5	±0.2	±0.2
Mean	G	277.5	514.6	717.0	17.7	152.0	170.0	1.4	3.1
S.D.		±35.0	±19.6	±13.2	±15.8	±12.3	±18.2	±0.2	±0.3
Controls									
Mean		165.5	611.0	732.8	16.9	152.7	169.6	1.78	3.20
S.D.		33.3	30.8	9.6	7.6	7.9	6.2	0.3	0.3

*meq./100 Gm. dry fat-free solids.
†Gm./100 Gm. dry fat-free solids.

TABLE VI. ANALYSIS OF SERUM AND MUSCLE TISSUES AFTER TREATMENT

PATIENT	SITE		PH	CO_2 (MM./L)	H_2O (GM.)	CL^- (MEQ.)	NA^+ (MEQ.)	K^+ (MEQ.)	N (GM.)	FAT (%)
		S*	7.5	31.9	914.6	95.4	144.6	2.9		
xD. N.	D†	M								
	G‡	M*			800.3	36.7	56.8	61.5	21.2	2.4
		S	7.4	25.6	915.6	95.4	131.0	3.6		
A. O.	D	M			786.9	25.7	54.5	73.6	27.9	1.2
	G	M			738.8	20.9	41.9	83.7	26.7	1.1
		S	7.4	27.2	910.8	97.8	128.5	3.1		
J. O.	D	M			785.1	22.8	38.7	82.5	28.0	1.3
	G	M			799.9	27.0	40.0	83.1	26.1	3.7
		S	7.5	30.9	913.0	90.8	127.0	3.1		
F. B.	D	M			785.0	20.0	39.0	96.0	30.0	1.2
	G	M			805.0	26.5	57.6	77.9	26.4	1.3
Mean		S	7.4	28.9	913.5	94.8	132.8	3.2		
S.D.				3.0	2.6	2.9	8.1	0.3		
Mean	D				785.7	22.8	44.1	84.0	28.6	
S.D.					1.1	2.9	9.0	11.3	1.2	
Mean	G				786.0	27.8	49.1	76.6	25.1	
S.D.					31.6	6.6	9.4	10.4	2.7	

*=Serum.
†=Deltoid muscle.
‡=Gastrocnemius muscle.
x=No specimen.

in the control, whereas there is no significant difference between the samples of gastrocnemius and deltoid. The values for the percentage of water in the intracellular phase were: for gastrocnemius $(H_2O)C = 71.70 \pm 1.32$ per cent; for deltoid $(H_2O)C = 72.8 \pm 1.1$ per cent, and in the control $(H_2O)C = 73.28 \pm 0.96$ per cent.

When extracellular fluid is recalculated in terms of tissue solids, as illustrated in Table VIII and in Fig. 3, the value for gastrocnemius muscle (304.2 ± 66.2) is markedly increased over the normal control value (165.5 ± 33.3) and is increased above the value for deltoid muscle (241.5 ± 39.6).

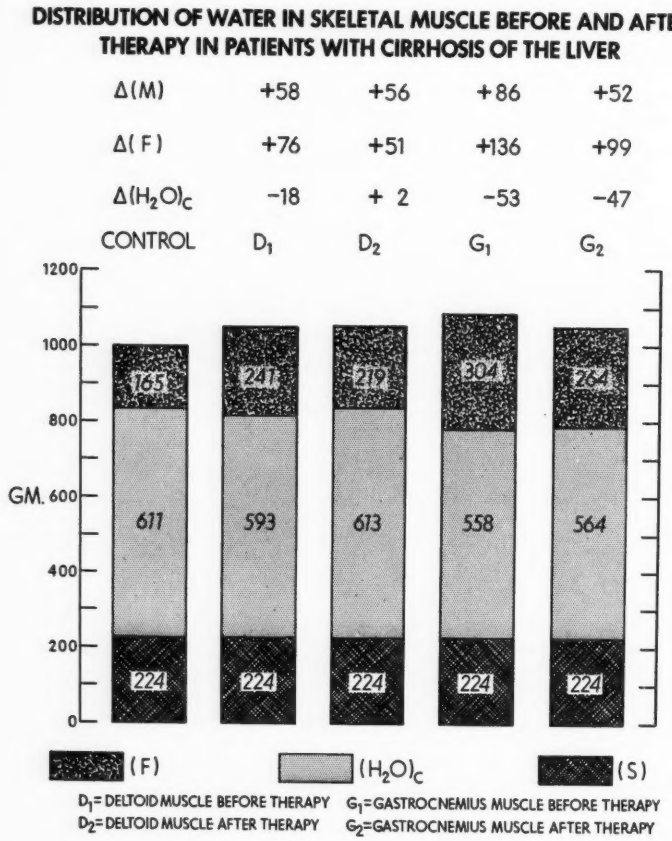

Fig. 3.—Extracellular phase (F). Intracellular water $(H_2O)c$ and solids (S) in control subjects and in edematous patients before and after treatment. The first column at the extreme left represents the mean values observed in control subjects. The next two columns represent the mean values for deltoid muscle before and after the loss of obvious edema and ascites (D_1 and D_2). The next two columns represent mean values for gastrocnemius muscle before and after treatment (G_1 and G_2). △ M represents the absolute change in one kilogram of fresh fat-free muscle as referred to control values; △ (F) and △ $(H_2O)c$ represent the absolute change in the extra and intracellular fluid volume per kilogram of fresh fat-free tissue as referred to the controls. Note that in each instance the values for (F) and $(H_2O)c$ are derived by correcting the values recorded in Tables IV and VI to a constant value for fat-free tissue solids (S), represented by the mean of the control observations.

At the conclusion of the study the total content of water decreased slightly in the gastrocnemius muscle but not in the deltoid muscle, the values being 786 ± 31.6 Gm. and 785.7 ± 1.1 Gm., respectively. This reduction in total muscle water represented a decrease in extracellular fluid in the specimens of gas-

TABLE VII. DERIVED ELECTROLYTE AND WATER DATA AFTER TREATMENT

PATIENT	SITE	$(H_2O)F$ (GM./KG.) FAT-FREE	$(H_2O)C$ FRESH TISSUE	$(H_2O)C$ (GM./KG.) CELLS	(NA) (MEQ./KG.)	(K) INTRACELLULAR	(NA + K) H_2O	RATIO: $(NA)M^*/(CL)M^*$	RATIO: $(K)M^*/(N)M^*$
D. N.	xD								
	G	334.1	468.2	706.6	12.4	129.2	141.6	1.5	2.9
A. O.	D	235.5	551.3	723.4	30.0	131.9	161.9	2.1	2.6
	G	190.9	547.3	678.7	18.8	151.5	170.3	2.0	3.0
J. O.	D	187.7	597.4	737.2	24.5	137.0	161.5	1.7	3.0
	G	222.3	577.6	744.8	17.7	144.3	162.0	1.6	3.2
F. B.	D	190.7	593.8	735.4	17.8	160.6	178.4	2.0	5.2
	G	252.5	551.9	740.9	33.7	139.7	173.4	2.2	4.2
Mean	D	204.6	580.8	732.0	24.1	143.2	167.3	1.9	3.6
S.D.		26.8	25.6	7.5	6.1	15.3	9.6	0.2	1.4
Mean	G	250.0	536.3	717.8	20.7	141.2	161.8	1.8	3.3
S.D.		61.5	47.3	31.2	9.1	9.3	14.3	0.3	0.6

D=Deltoid.
G=Gastrocnemius.
S.D.=Standard deviation.
x=No specimen.
*meq./100 Gm. dry fat-free solids.

TABLE VIII. PHASE MASS DATA FOR SKELETAL MUSCLE

PATIENT		(M) (GM.)	(F) (GM.)	$(H_2O)C$ (GM.)	(S) (GM.)
D. N. (Deltoid)	Initial	1000	165	611	224
	Final	1000	276	518	206
	$(M)_f$	1087	300	563	224
	Δ	+87	+135	−48	
D. N. (Gastroc)	Initial	1000	165	611	224
	Final	1000	335	484	181
	$(M)_f$	1238	415	599	224
	Δ	+238	+250	−12	
Mean* (Deltoid)	$(M)_f$	1058	242	594	
	S. D. =	±45	±40	±35	
	Δ	+58	+77	−17	
Mean† (Gastroc)	$(M)_f$	1086	304	558	
	S. D.	±101	±66	±41	
	Δ	+86	+139	−53	

(M)=Extracellular phase plus intracellular phase.
(F)=Extracellular phase.
$(H_2O)c$=Intracellular water.
(S)=Tissue solids.
Initial=The mass of the phases of one kilogram of muscle from the control patients.[2]
Final=The mass of phases of 1 kg. of muscle from the cirrhotic patient with edema and ascites.
$(M)_f$=Absolute final weight of the initial phases of muscle after the development of edema.
Δ=The difference between normal values and the absolute values following the development of edema $(M)_f$.
*Mean values for all samples of deltoid muscle before treatment.
†Mean values for all samples of gastrocnemius muscle before treatment.

trocnemius muscles, the values being 250.0 ± 61.5 Gm. of extracellular fluid per kilogram of fresh fat-free tissue and for deltoid 204.6 ± 26.8 Gm. of extracellular fluid per kilogram of fresh fat-free tissue. In contrast, intracellular water did not change significantly in either the gastrocnemius or the deltoid muscles. When these values are calculated in terms of tissue solids, the extracellular fluid in gastrocnemius muscle decreased significantly, whereas the values for deltoid muscle remained approximately the same (Fig. 3).

Potassium and nitrogen: The potassium content of skeletal muscle, referred to fresh fat-free tissue, was less than normal in the samples of gastrocnemius and deltoid muscle obtained before treatment (Table IV). This point of reference may be open to question in the presence of edema, since a significantly greater proportion of tissue consists of low-potassium fluid (extracellular edema). When potassium is referred to nitrogen (Table V) the K:N ratio does not differ significantly from the control value in normal muscle. Following therapy, the tissue potassium content as referred to fresh fat-free muscle changed only slightly (Table VI). The K:N ratio (Table VI) increased slightly both in gastrocnemius and deltoid muscles; these differences are probably not significant.

Sodium and chloride: Both sodium and chloride were increased in the samples of gastrocnemius and deltoid muscle (Table IV). These increases are significant both when referred to fresh fat-free tissues and to fat-free solids. The ratio of sodium to chloride in the tissues was decreased in both deltoid and gastrocnemius muscles preceding therapy as compared with the control and reflects a greater increase of tissue chloride than of tissue sodium in the edematous patients. Following therapy and the relief of obvious edema, the tissue chlorides decreased in both gastrocnemius and deltoid muscles. Simultaneously, the mean value for sodium increased in both gastrocnemius and deltoid muscles. It is of interest that in three instances tissue sodium increased, whereas in one patient (F. B.) this value diminished. As a result, the Na:Cl ratio increased with therapy and at this new level did not differ significantly from the control value.

Intracellular sodium and potassium: The concentration of intracellular sodium did not differ significantly from the control values, either in the deltoid or gastrocnemius muscles (Table V). Similarly, pretreatment concentrations of intracellular potassium and of the sum of sodium and potassium were not significantly different in the edematous cirrhotics and in control subjects. Following treatment, intracellular sodium concentration increased slightly and intracellular potassium decreased slightly. The net result of these fluctuations produced no significant changes in the sum of intracellular sodium and potassium. The observed alterations in the individual concentrations of sodium and potassium as related to control values in the pre- and posttreatment samples probably are not significant. It is important to recognize that the relative quantities of active and inactive cell base cannot be assessed by this technique.

Red blood cell data: The intracellular red cell concentrations of chloride, sodium, and potassium were calculated for each of the blood samples analyzed. Significant alterations in these moieties were not observed before, during, or after therapy; hence, the extensive data on intrared cell electrolytes are not included in this report.

Precautions in the interpretation of the balance data: The cation exchange resin diverted a considerable portion of ingested electrolytes into the feces. As a result, fecal sodium and potassium constituted a major factor in the balance data. Collections and analyses of feces are seldom as accurate or dependable

as are collections and analyses of urine. However, by assiduously careful technique and by constant supervision of the collection and preparation of the stool samples, errors from these sources probably have been minimized.

The use of the external chloride balance as a reference for the calculation of shifts in body fluids is open to criticism on theoretical grounds, since it is well known that a significant quantity of the body chloride is intracellular or transcellular.[30] Similarly, phase-mass data derived from sodium and/or potassium balances require careful scrutiny, because large quantities of these ions are stored in connective tissue, bone, and cartilage and thus do not enter into prompt equilibrium with the cations of the serum and extracellular phase.[31] Conclusions drawn from balance data must be interpreted further in relation to the fact that the loss of electrolytes and water through the skin cannot be measured precisely by any dependable technique at present. In addition, the cyclic variations in electrolyte balances recognized in normal individuals occur also in edematous patients.

DISCUSSION

The present investigation provides evidence indicating that the edema and ascites in cirrhosis of the liver represent an isotonic expansion of the extracellular phase. This is demonstrated by the metabolic balance data in that the actual weight loss corresponded closely to the loss of extracellular fluid. Further, the results of the skeletal muscle analyses demonstrated that the increased tissue water content in edematous areas was exclusively extracellular. In a previous study it was demonstrated that when an increase in tissue fluid is hypotonic, both intracellular and extracellular phases are increased, whereas when such an increase is isotonic, only the extracellular fluid expands.[34]

External chloride balance was utilized as the point of reference in calculating changes in the volume of extracellular water. As indicated above, this method of estimating changes in the volume of the extracellular phase has been challenged on theoretical grounds; however, other methods for serially measuring changes in this moiety, such as the inulin, thiosulfate, or radiosulfate space, also are open to criticism either on practical, technical, or theoretical grounds. It is of interest, that when the sodium balance is utilized in calculation of the changes in extracellular fluid volume, the values obtained differ quite significantly from those achieved with the chloride balance. A similar discrepancy has been observed in some patients with congestive heart failure.[32, 33] Several factors must be considered with reference to this observation. A large quantity of the sodium involved in the current study was diverted into the fecal stream and may have been unaccounted for through inadvertent loss. Further, sodium loss through the skin by way of the sweat was not measured, and this factor would serve to account in part for the observed discrepancy. Another possible factor is the transfer of sodium from the extracellular phase to some compartment in which concentration equilibrium is less readily achieved. This may result from the movement of sodium into the intracellular phase or the storage of this ion in bone, cartilage, and connective tissue. In two of the patients (D. N., A. O.) the intracellular sodium concentration increased at the close of the study as compared with the initial values (Tables V and VII). In one subject (J. O.) intracellular sodium increased in the deltoid muscle but

decreased in the gastrocnemius, whereas in the fourth subject (F. B.) this value decreased in both deltoid and gastrocnemius muscles. Thus, in some instances an increase in intracellular sodium would seem to explain the discrepancy between the external losses of sodium and of chloride. It is important to recognize that in calculating the skeletal muscle data the value for "intracellular sodium" actually represents "excess" sodium or that quantity of tissue sodium not accounted for by the volume of extracellular water. With the methods currently available it is impossible to determine whether this "excess" sodium is bound to connective tissue or to the cell membrane, or whether it is actually in the intracellular phase.

Hyponatremia was observed in three of the four cirrhotic patients studied. This finding may be explained by dilution of the extracellular space, by transfer of sodium into the intracellular phase, or by movement of sodium into an immiscible pool. Clinical symptoms possibly referable to hyponatremia or electrolyte depletion developed in only one patient who manifested hypokalemia at the same time. This patient improved clinically after the ingestion of foods rich in potassium without the administration of additional sodium ion; which suggest that the hyponatremia did not represent actual depletion of body sodium but rather the transfer of sodium ions out of the extracellular phase. Hyponatremia in the presence of normal or increased total body sodium ("the sodium paradox") has been described in the postsurgical state[35] and more recently has been observed in patients with congestive heart failure and in those with cirrhosis of the liver.[36] The occurrence of hyponatremia in the presence of an apparently isotonic expansion of the extracellular phase seems paradoxical. Studies correlating serum sodium concentration with total serum osmotic activity have demonstrated that although the sodium ion plays a major role in determining the serum osmotic activity, other factors as yet unidentified may influence the total osmotic strength.[36] Thus hyponatremia can occur in the presence of an isotonic extracellular fluid. It is apparent, therefore, that despite the discrepancies between the external balances of sodium and of chloride and despite the occurrence of hyponatremia, the data suggest that the edema associated with cirrhosis of the liver is essentially extracellular.

The occurrence of abnormal sodium and water metabolism in patients with cirrhosis of the liver without ascites and edema has been observed previously.[37] In the present study the increased volume of extracellular fluid persisting after the disappearance of clinical signs of ascites and edema is in accord with this concept and suggests that the factor or factors responsible for the formation of ascites and edema in cirrhosis of the liver are still operative.

SUMMARY

Four patients with proved cirrhosis of the liver complicated by ascites and edema were studied by the metabolic balance technique under the following conditions: (1) a control period with an intake of 3.0 Gm. of sodium chloride; (2) a similar diet with 45 Gm. of cation exchange resin added daily; (3) similar diet but with an intake of 1.5 Gm. of sodium chloride per day, together with 45 Gm. of cation exchange resin; and (4) final period comprising the conditions of the third period and the administration of a mercurial diuretic

on alternate days. Samples of deltoid and gastrocnemius muscle were obtained from six cirrhotic patients. Four of these served as subjects for the metabolic studies and in these biopsies of the deltoid and gastrocnemius muscles were obtained at the close of the study.

The balance data were utilized to partition the weight loss which occurred as a result of the therapy. It was observed that the edema fluid lost was extracellular in composition.

The samples of gastrocnemius and deltoid muscles obtained before therapy contained an increased amount of extracellular water without significant alteration in intracellular water; the fluid content of the gastrocnemius exceeded that of the deltoid, reflecting the influence of gravitational factors. After therapy the volume of extracellular fluid in the gastrocnemius muscle decreased but was unchanged in the deltoid; both remained greater than normal.

These observations indicate that the mechanism or mechanisms responsible for the formation of ascites and edema in cirrhosis of the liver must be capable of producing an isotonic increase in the volume of extracellular fluid.

CONCLUSIONS

The edema and ascites in cirrhosis of the liver represent essentially isotonic expansions of extracellular fluid. With the methods utilized in this study, intracellular electrolytes and water and intracellular osmolarity were not altered significantly. The elimination of edema and ascitic fluid during therapy reflected the movement of isotonic extracellular fluid. The pattern of loss of edema fluid was similar during treatment with a low salt diet, exchange resins, and with mercurial diuretics. The decrease in body weight during "sodium elimination" therapy in patients with cirrhosis of the liver with edema and ascites corresponded very closely with decreases in the volume of extracellular fluid. Tissue overhydration persists despite the disappearance of overt edema and ascites, indicating persistence of the causes of the fluid retention. The hyponatremia and hypokalemia in cirrhosis of the liver with edema and ascites remain unexplained. The pathogenesis of the edema and ascites in cirrhosis of the liver appears to depend upon a mechanism capable of inducing isotonic expansion of the extracellular phase of body water.

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INVESTIGATION OF TRANSFER RATES OF ALBUMIN TAGGED WITH I^{131} IN ASCITES AND EDEMA

II. Studies in Control Subjects and Patients With Cirrhosis

James A. Schoenberger, M.D., George Kroll, M.D., Edward L. Eckert, and Robert M. Kark, F.R.C.P.

Chicago, Ill.

In a previous communication,[1] a method was presented for quantitatively estimating the exchange rate of albumin from the vascular compartment to the peritoneal cavity in patients with ascites due to Laennec's cirrhosis. It was also shown that the plasma and ascitic-fluid albumin were in a state of dynamic equilibrium, confirming in man a finding first demonstrated in animals by Yuile and associates.[2] The present study was undertaken with two objectives in mind: first, to compare vascular endothelial and peritoneal cavity albumin transfer rates in control subjects with those in patients with cirrhosis; and second, to observe serial changes in patients with cirrhosis during the course of therapy. Similar studies have been carried out by Berson and associates.[3, 4]

METHODS

Subjects.—The control subjects were ten patients who had been admitted to the hospital for illnesses entirely unrelated to a state of edema or ascites. None had cardiovascular, renal, hepatic, or hormonal disease; nor did they give a history of edema at any time in the past. The specific diagnosis, age, and sex of each patient is shown in Table I. The control subjects consumed a general ward diet and were allowed to be up and about the ward.

Patients ill with ascites as a result of Laennec's cirrhosis were studied in an air-conditioned metabolic ward. The diagnosis was established in each case by a typical clinical history, including alcoholism, by physical findings and by biochemical studies. In one case, a needle biopsy of the liver was obtained to confirm the clinical findings. The diet of each patient consisted of a fixed daily intake of 2.5 Gm. of protein per kilogram body weight, twice the calculated basal caloric requirements, less than 500 mg. of sodium, and 2,500 ml. of fluid. Detailed descriptions of the diets have been published elsewhere.[5] Patients were allowed to be up and about the ward. A total of 6 patients with cirrhosis was studied, and in 3 of these, 2 or more serial observations were made.

Procedure.—A detailed description of the procedure and the mathematical analysis of the results have been published elsewhere.[1] The patients were given Lugol's solution before and during each study in order to prevent the uptake of I^{131} by the thyroid gland. All were in a "steady state" throughout each 10- to 14-day period of study, as shown by constant weight and insignificant changes in concentration of serum and ascitic albumin.

From the Department of Medicine and the Research and Educational Hospitals of the University of Illinois, and the Presbyterian Hospital, Chicago, Ill.

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A tracer dose (50 to 80 μc) of I^{131} bound to 5 to 10 mg. of albumin* was injected either intravenously or intraperitoneally. At frequent intervals thereafter samples of plasma and ascitic fluid were obtained for analysis of radioactivity. Dried samples were counted either in a thin end-window GM counter, in early experiments, or in a windowless gas flow counter. After appropriate corrections for physical and biologic decay and self absorption, radioactivity per gram of albumin was plotted on a logarithmic scale, against time on a linear scale.

When the data were represented in this manner, the disappearance of radioactivity from the plasma, after intravenous injection, was found to follow certain general relationships, which are shown by a typical experiment depicted in Fig. 1. Plasma radioactivity fell rapidly in a curvilinear manner during the first forty-eight to seventy-two hours after injection and then slowly at a

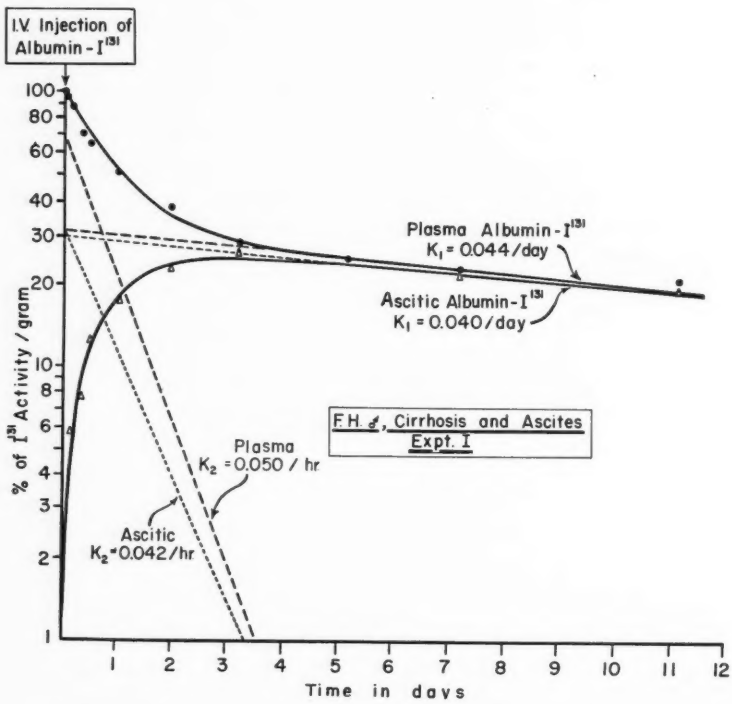

Fig. 1.—Plasma and ascitic fluid radioactivity following intravenous injection of albumin I^{131} in Patient F. H., ill with cirrhosis and ascites. Radioactivity is shown on a semilogarithmic scale on the ordinate as the per cent of the injected radioactivity per gram of albumin, assigning the value 100 per cent to the fifteen-minute plasma sample. (See text for discussion.)

linear rate for as long as observations were continued. The slope of the straight-line portion of the curve was considered to represent the metabolic decay rate of albumin I^{131}.[6] More recent work by Berson and associates[3] has suggested that this assumption is not entirely valid since variable amounts of rapidly degrading albumin have been shown to be present in some lots of labeled albumin. The first curvilinear phase of the plasma curve was assumed to represent two simultaneous processes: first, the transfer of the tagged albumin

*Abbott Laboratories, North Chicago, Ill.

from the circulation into the exchangeable albumin pool; and second, the metabolic decay of the albumin. The straight decay line was extrapolated to the time of complete mixing (or time of injection) and values along this extrapolated line were subtracted from the actual curve. This procedure yielded a series of points which fell on a straight line, indicating that the transfer could also be represented by an exponential function. The actual curve of disappearance of radioactivity thus consisted of 2 experimental functions. The slopes of the straight lines were obtained by graphic analysis of radioactivity in the plasma.

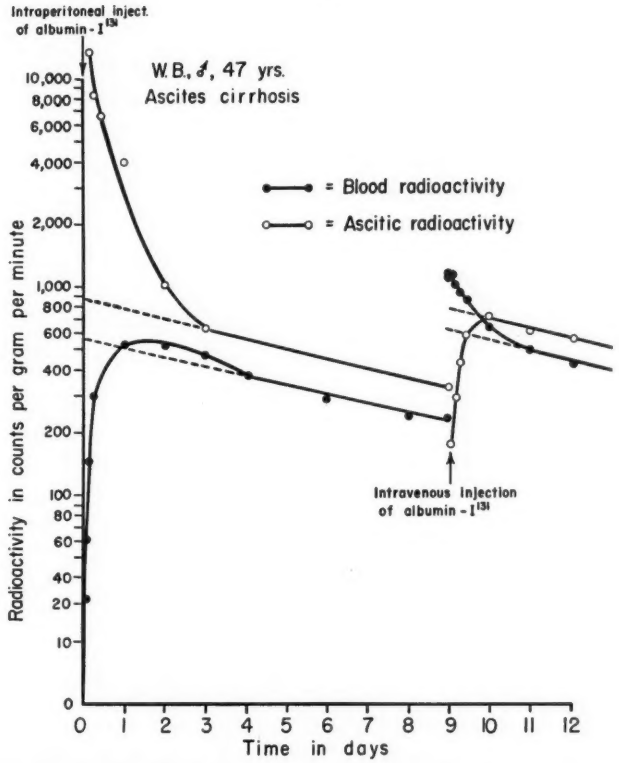

Fig. 2.—Plasma and ascitic fluid radioactivity in Patient W. B., ill with cirrhosis and ascites; first, following intraperitoneal injection of albumin I^{131}; and second, following intravenous injection nine days later. In this figure, radioactivity is plotted as counts per gram of albumin per minute.

Following intravenous injection, the radioactivity increased rapidly in a curvilinear fashion in the ascitic fluid until distribution equilibrium with plasma occurred. It then decreased at a linear rate equal to that of the plasma disappearance rate. Radioactivity of plasma and ascitic fluid after distribution equilibrium was reached was proportional to the concentration of albumin in these two compartments. It was also assumed that ascitic fluid activity, until equilibrated with plasma, reflected a transfer process into ascitic fluid from plasma, combined with simultaneous metabolic degradation. After equilibrium, metabolic decay predominated. Thus the complex curve of ascitic fluid radioactivity

was also assumed to be a two-term exponential curve, and the slopes of the straight lines were analyzed in a manner similar to analyses of the plasma curves. In these analyses it was assumed that the exchange of albumin between plasma and ascitic fluid involved only the peritoneal and vascular compartments. When labeled albumin was injected intraperitoneally, the plotted curves were similar in shape to those described above except that the specific activity decreased in the ascitic fluid and increased in the plasma. True distribution equilibrium did not occur during the course of observation, which exceeded nine days (Fig. 2).

In several important instances there were exceptions to these general relationships. These will be indicated below in the section on "Results."

The fifteen-minute plasma sample was assumed to represent the maximum radioactivity in the plasma at the moment of complete mixing of the injected and circulating albumin. Initially, there was no radioactivity in the ascitic fluid, and within the first fifteen minutes required for equilibration in the plasma, the amount of albumin I^{131} entering the ascitic fluid was assumed to be negligible. Hence, the first portion of the diffusion curves represented a unidirectional transfer of labeled albumin from the plasma. Using mathematical formulae already published,[1] the derivatives of the diffusion curves at fifteen minutes were used to calculate the rate of disappearance of tagged albumin from the plasma, or its rate of appearance in the ascitic fluid. In these studies, tagged albumin was considered to behave in an identical fashion to endogenous albumin[6-8]; therefore, the same rate of transfer applied both to tagged and untagged albumin. Since the patients were in a "steady state," the rate of transfer of endogenous albumin out of the ascitic fluid equaled its rate of transfer into the peritoneal compartment.

From the data, additional information was also obtained: the total body pool of albumin, the mass of extravascular albumin, the plasma volume, and the mass of circulating albumin.[6]

RESULTS

1. Fate of Intravenously Injected Albumin I^{131} in Control Subjects.—Table I summarizes the data obtained in control subjects. On the average, in the 10 subjects, the half-time for distribution of tagged albumin was 13.3 hours, but there were 3 subjects with distinctly higher values of 15.8, 15.9, and 22.3 hours, respectively. Tagged albumin disappeared from the plasma at an average rate of 3.1 per cent per hour, indicating that endogenous albumin exchanged at a rate of 2.9 Gm. per hour. In 9 subjects, the total albumin pool averaged 216 Gm., of which 120 Gm. were extravascular.

Analysis of the data from this group of control subjects disclosed that the assumption of a single rate of transfer was not entirely valid. Similar conclusions were reached by Berson and co-workers[4] in their studies. When the extrapolated decay rate was subtracted from the observed curve, the series of points which were obtained did not fall on a straight line. This was true for points obtained during the first few hours after injection, when the rate of fall was more rapid. Thereafter, the calculated points fell on a reasonably

straight line. Unfortunately, in 7 of the 10 subjects, the number of blood samples which could be measured during the first 24 to 48 hours was limited. Therefore, the best possible line was drawn by visual inspection, with the use only of points occurring later than the first few hours after injection. This was done even though it was obvious that a single exponential term failed to characterize completely the distribution of tagged albumin out of the plasma. An example of a typical experiment is shown in Fig. 3.

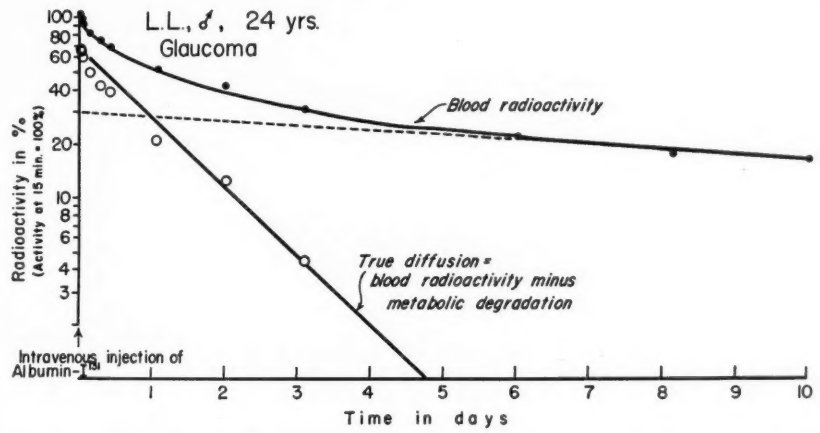
L.L., ♂, 24 yrs.
Glaucoma
Blood radioactivity
True diffusion = blood radioactivity minus metabolic degradation
Radioactivity in % (Activity at 15 min. = 100%)
Intravenous injection of Albumin-I131
Time in days

Fig. 3.—Plasma radioactivity in control subject L. L. following intravenous injection of albumin I^{131}. Radioactivity is plotted as in Fig. 1. Note the deviation of the points from the diffusion curve, most marked in the first hour after injection.

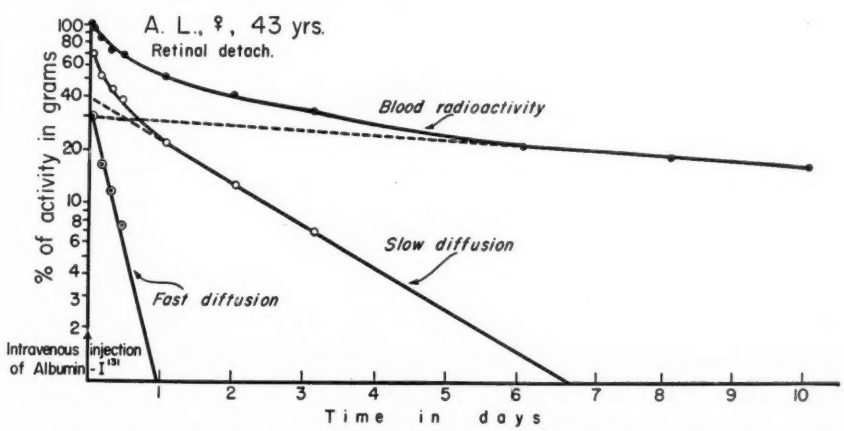

Fig. 4.—Plasma radioactivity in control subject A. L. following intravenous injection of albumin I^{131}. Radioactivity plotted as in Fig. 1. (See text for discussion of figure.)

These results indicated that a single straight line does not accurately express the rate at which tagged albumin leaves the circulation, and, therefore, any over-all average rate calculated from a single exponential term is subject to considerable error (Table I).

In 3 of these subjects (M. B., A. L., and L. L.) the points obtained by subtraction of the metabolic decay rate from the observed curve indicated more

TABLE I. THE FATE OF ALBUMIN I^{131} INJECTED INTRAVENOUSLY IN CONTROL SUBJECTS

NO.	PATIENT	AGE	SEX	DIAGNOSIS	SINGLE TRANSFER CURVE				METABOLIC DECAY CURVE				
					K OR SLOPE PER HR.	HALF-TIME (HR.)	%/HR.	GM./HR.	K OR SLOPE PER HR.	ALB. POOL (GM.)	EXTRA-VASCULAR ALB. (GM.)	PLASMA VOL. (ML.)	CIRCULATING ALB. (GM.)
1.	P. R.	50	F	Cataract	0.0693	10.0	3.9	2.6	0.0055	151	85	2,140	66
2.	C. H.	56	M	Keratitis, glaucoma	0.0666	10.4	3.9	4.2	0.0045	261	154	3,590	107
3.	M. B.	43	F	Glaucoma	0.0546	12.7	3.2	--	0.0048	---	---	---	---
4.	C. M.	38	M	Low back ache	0.0495	14.0	2.3	2.8	0.0044	227	104	3,219	123
5.	R. S.	28	M	Ocular foreign body	0.0436	15.9	2.7	2.5	0.0030	243	153	2,386	90
6.	W. K.	40	M	Retinal detachment	0.0630	11.0	2.5	4.5	0.0036	302	119	5,303	183
7.	M. D.	34	F	Uterine fibroid	0.0693	10.0	4.3	2.9	0.0024	178	111	2,208	67
8.	W. W.	52	M	Duodenal ulcer	0.0636	10.9	3.5	3.8	0.0044	243	136	3,213	107
9.	A. L.	43	F	Retinal detachment	0.0311	22.3	2.1	1.2	0.0027	185	127	1,658	58
10.	L. L.	29	M	Glaucoma	0.0439	15.8	2.7	1.6	0.0036	155	95	2,343	60
				Mean	0.0555	13.3	3.1	2.9	0.0039	216	120	---	---

definitely that the distribution curve did not possess a single rate of change. Since the curve fell rapidly in a curvilinear manner in the first eight or so hours and then slowly in a linear fashion, it was apparent that there were 2 or more rates for the distribution process: a rapid and slow phase. The slow rate was subtracted from the curvilinear portion of the diffusion curve after extrapolation of the straight portion of the curve back to the time of complete mixing. The points, usually 3 or 4 in number, obtained by this second subtraction were also found to fall on a straight line, so that, in these 3 instances, distribution was separable into a two-term exponential function. The straight-line portions of these curves were constructed by the method of least squares. The results of this analysis are shown for one control subject, A. L., in Fig. 4. In this case the observed curve was composed of three exponential terms; i.e., rapid and slow rates of distribution, and metabolic decay. The results of analysis of the two phases are shown in Table II. Included in the table are results for a cirrhotic patient, F. H., in whom the data also permitted identification of two transfer rates. In the remainder of the studies on both control subjects and cirrhotic patients, the data were too few to allow separation of the initial rapid disappearance of tagged albumin from plasma into 2 rates.

The data derived from double phase curves (Table II) were too few to justify calculation of averages. In the control subjects, a half-time varying from 0.9 to 4.8 hours was found for the rapid transfer phase. Tagged albumin disappeared from the plasma at a rate of 4.3 to 11.8 per cent per hour. The rapid rate of transfer for endogenous albumin back and forth from the plasma was 2.6 and 9.8 Gm. per hour in 2 subjects. The slower phase had a half-time of 16.6 to 30.8 hours. Tagged plasma albumin disappeared at a rate of 0.9 to 1.7 per cent per hour, and in 2 subjects, 0.5 and 1.5 Gm. per hour of endogenous albumin was transferred in and out of the plasma. In one patient with cirrhosis, the results, shown in Table II, fell within the ranges found in the control subjects. From the data it was also possible to estimate the size of each of two albumin pools which equilibrated either rapidly or slowly with plasma. These estimates are significant only with respect to order of magnitude, because, from the data alone, it is impossible to state that the two exponents whose intercepts were used for the estimates represent only an equilibration process. Subject to these reservations, the calculations* indicated a rapidly exchangeable pool in two control subjects of 10.0 and 25.0 Gm. or 7.8 and 20 per cent, respectively, of the total extravascular albumin. For the slowly equilibrating pools, values of 101 and 117 Gm. (80.0 and 92.2 per cent of the total extravascular pool) were found. In the patient with cirrhosis, in 2 separate experiments, 26 and 31 Gm. were in the albumin pool with rapid equilibration. This constituted 20.7 and 29.5 per cent, respectively, of the total extravascular pool of albumin.

*This was calculated as follows:

$$\frac{100\%}{100\% - \text{fast diffusion intercept}} \times \text{plasma albumin} = \text{rapid distribution albumin mass (which includes plasma albumin)}$$

Rapid distribution albumin mass − plasma albumin = rapid equilibrating albumin pool
Total exchangeable albumin pool − rapid distribution albumin pool = slowly equilibrating albumin pool.

TABLE II. FATE OF ALBUMIN I^{131} INJECTED INTRAVENOUSLY
(SEPARATION OF TRANSFER RATES INTO RAPID AND SLOW PHASES)

		RAPID PHASE						SLOW PHASE					
NO.	PATIENT	K/HR.	T½/HR.	%/HR.	GM./HR.	TOTAL GM.	% EXTRA-VASCULAR ALB.	K/HR.	T½/HR.	%/HR.	GM./HR.	TOTAL GM.	% EXTRA-VASCULAR ALB.
1*	M. B.	0.21	3.4	4.3	--	--	19.0	0.042	16.6	1.5	--	---	81.0
2*	A. L.	0.15	4.8	4.4	2.6	25	20.0	0.023	30.8	0.9	0.5	101	80.0
3*	L. L.	0.78	0.9	11.8	9.8	10	7.8	0.037	18.6	1.7	1.4	117	92.2
4† b	F. H.	0.30	2.3	9.5	6.5	31	20.7	0.044	16.0	1.7	1.1	119	79.3
c		0.43	1.6	15.1	7.3	26	29.5	0.029	24.0	0.9	0.44	62	70.5

*Control Subject.
†Patient with cirrhosis; two studies five and one-half months apart are shown.

2. Fate of Intraperitoneally Injected Albumin I^{131} in Control Subjects.—Albumin I^{131} was injected intraperitoneally in three control subjects. In two, this was done after establishing a small pneumoperitoneum, and, in the third, by posterior colpocentesis. Fig. 5 shows the curves of concentration of radioactivity in the plasma following injection. The general resemblance of this curve to those obtained following intravenous injection (Fig. 1) is apparent. Unfortunately, it was impossible to establish the rate at which tagged albumin (and hence endogenous albumin) left the peritoneal cavity because we could not obtain the volume of peritoneal fluid or the concentration and the specific radioactivity of the albumin in that fluid. However, the data demonstrate that albumin diffuses out of the normal peritoneal cavity into the plasma in a manner similar to that which occurs in patients with cirrhosis and ascites (Fig. 2).

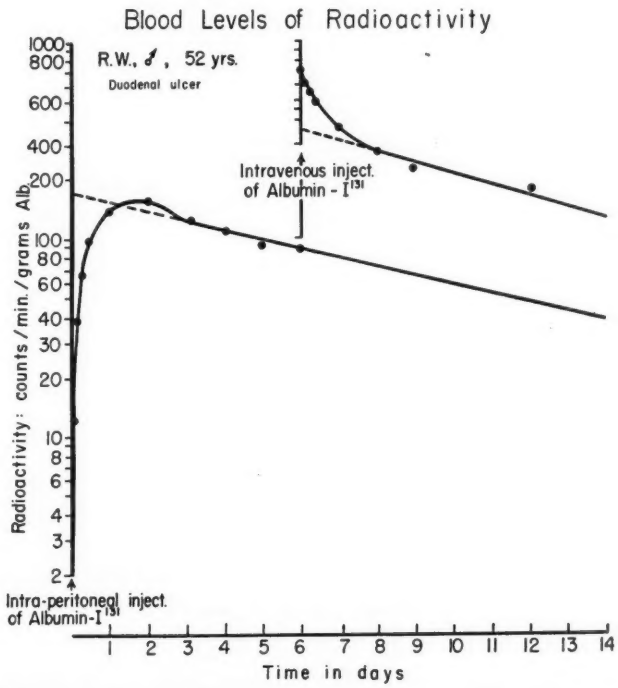

Fig. 5.—Plasma radioactivity in control subject R. W. following intraperitoneal injection "after pneumoperitoneum" of albumin I^{131}. Radioactivity is plotted as in Fig. 2. Six days later the subject received a second injection by the intravenous route.

3. Fate of Intravenously Injected Albumin I^{131} in Patients With Cirrhosis.—Six patients with Laennec's cirrhosis and ascites were studied when they were in a "steady state." No patient had an appreciable change in body weight or serum albumin concentration during the ten- to fourteen-day period required for the study. The data of these patients are summarized in Table III. In all cases, the best possible single transfer rate was determined, although as in the case of the control subjects, there appeared to be more rapid transfer

TABLE III. THE FATE OF ALBUMIN I^{131} INJECTED INTRAVENOUSLY IN PATIENTS ILL WITH LAENNEC'S CIRRHOSIS

				SINGLE TRANSFER CURVE				METABOLIC DECAY CURVE				
NO.	PATIENT	AGE	SEX	K OR SLOPE	HALF-TIME (HR.)	%/HR.	GM./HR.	K OR SLOPE	ALB. POOL (GM.)	EXTRA-VASCULAR ALB.	PLASMA VOL.	CIRCULATING ALB.
1	W. B.	47	M	0.0648	10.7	3.6	3.8	0.0040	249	144	3,655	105
2	R. S.	45	M	0.0626	11.5	3.7	5.0	0.0035	354	137	5,114	217
3	R. E.	53	M	0.0379	18.3	2.3	1.6	0.0022	199	129	3,526	70
4a	H. L.	41	M	0.0582	11.9	3.9	2.5	0.0036	192	126	2,430	66
b	5 mo. later			0.0651	10.6	4.3	4.3	0.0032	296	194	3,457	102
5a	S. G.	47	M	0.0960	6.6	4.4	7.1	0.0035	300	138	5,762	162
b	3 mo. later			0.0914	6.3	4.8	5.4	0.0030	276	154	4,588	122
6a	F. H.	42	M	0.0498	13.9	3.4	2.2	0.0018	203	139	3,950	64
b	2½ mo. later			0.0573	12.1	4.0	2.7	0.0018	219	150	3,905	69
c	8 mo. after 1st study			0.0519	13.4	3.5	1.7	0.0026	146	98	2,114	48
	Mean (Initial studies only)			0.0616	12.2	3.6	3.7	0.0031	250	136	---	---

of labeled albumin from the plasma in the first few hours after injection. In only one patient (F. H., Table II) did the data permit an accurate separation of two phases of distribution. In three of the patients (H. L., S. G., and F. H.,) studies were repeated after some months of treatment, and one patient (F. H.) was studied three times.

The average half-time for the diffusion phase was 12.2 hours; the average rate of disappearance of tagged albumin was 3.6 per cent, indicating a rate of transfer of 3.7 Gm. per hour. The total albumin pool averaged 250 Gm., of which 136 Gm. were extravascular.

Because of the inherent errors in using double exponential functions to depict the disappearance of tagged albumin from the plasma in a multicompartment system, no statistically valid comparison could be made between control and patient groups. Furthermore, comparison of data obtained on different occasions in the same patient was subject to the same errors. There were, however, definite changes in the sizes of the exchangeable albumin pools in those patients who were studied on two or more occasions. In patient H. L., the total albumin pool rose from 192 to 296 Gm., both circulating and extravascular albumin sharing in this increase. This patient had shown marked clinical improvement between the two studies as evidenced by increase in appetite and feeling of well-being, a spontaneous loss of ascites, and a gain in weight.

In patient S. G., the total albumin pool fell slightly because of a drop in the plasma volume and circulating albumin. He failed to respond to dietary therapy and had a larger ascitic fluid volume at the time of the second study. Total ascitic fluid albumin rose from 7.2 to 13.8 Gm. In patient F. H., total exchangeable albumin fell remarkably between the second and third studies despite the fact that he had made good clinical progress with a spontaneous diminution in ascitic fluid volume. During this time, the total ascitic fluid albumin also decreased, dropping from 68.4 to 49.1 to 9.3 Gm. in the three successive studies.

4. The Appearance of Albumin I^{131} in Ascitic Fluid Following Intravenous Injection and the Fate of Intraperitoneally Injected Albumin I^{131} in Patients With Cirrhosis.—Following intravenous injection of albumin I^{131}, the rate of transfer of tagged albumin into ascitic fluid was determined in six cirrhotic patients. In all cases, subtraction of the observed curve from the extrapolated decay curve yielded points which uniformly fell on a straight line (Fig. 1). The data are summarized in Table IV. In three of these patients, albumin I^{131} was injected intraperitoneally, usually after completion of the intravenous study. From an analysis of the ascitic fluid curve, the rate of transfer of tagged albumin out of the ascitic fluid was calculated and could be compared to the rate, determined after intravenous injection, for transfer into the ascitic fluid. Fig. 2 shows a typical study. Serial observations were made on 2 patients. In patient S. G., rates of 0.42 and 0.40 Gm. per hour were found initially and after three months of treatment, respectively. Patient F. H. had a rate of 0.62 Gm. per hour initially. After two and one-half months of therapy, the rate was 0.51 Gm. per hour, and there was minimal clinical im-

TABLE IV. THE APPEARANCE OF ALBUMIN I^{131} IN ASCITIC FLUID FOLLOWING INTRAVENOUS INJECTION AND THE FATE OF INTERPERITONEALLY INJECTED ALBUMIN I^{131} IN PATIENTS WITH CIRRHOSIS

		INTRAVENOUS INJECTION				INTRAPERITONEAL INJECTION				
NO.	PATIENT	ASCITIC VOLUME T-1824 IN LITERS	K OR SLOPE PER HR.	HALF-TIME (HR.)	GM./HR.	ASCITIC VOLUME ALB. I^{131}	ASCITIC VOL. T-1824	K OR SLOPE PER HR.	HALF-TIME (HR.)	GM./HR.
1	W. B.	-	0.092	7.5	-	-	-	-	-	-
2	R. S.	12.1	0.014	50.5	0.65	8.0	10.3	0.0044	158.0	0.31
3	R. E.	9.47	0.048	14.4	0.63	4.94	5.34	0.0375	18.5	0.58
4	H. L.	-	0.224	3.9	-	-	-	-	-	-
5a	S. G.	0.89	0.110	6.3	0.42	-	-	-	-	-
b		1.95	0.068	10.2	0.40	-	-	-	-	-
6a	F. H.	6.16	0.042	16.5	0.62	-	-	-	-	-
b		6.54	0.038	18.4	0.51	-	-	-	-	-
c		1.15	0.064	10.8	0.25	1.048	1.37	0.36	19.3	0.30

provement accompanying this. Eight months later, the rate was only 0.25 Gm. per hour and marked clinical improvement was apparent, as indicated previously.

In the three patients (R. S., R. E., and F. H.) in whom the albumin I^{131} was injected intraperitoneally, the rate of transfer of albumin as calculated from the distribution curve of tagged albumin agreed reasonably well in 2 cases (R. E. and F. H.) with the rate calculated after intravenous injection.

DISCUSSION

The data in 4 experiments confirm the existence of 2 rates of distribution between plasma and extravascular space for tagged albumin given intravenously as reported recently by Berson and associates.[3] The latter group observed mean half-times of three and twenty-four hours. These values compare favorably with our findings. It is possible, however, that several pools of extravascular albumin exist in the body, and it is likely that a family of curves would be required to characterize completely the disappearance of injected tagged albumin from the plasma. Further investigation of this aspect is in progress using more sensitive methods of detecting radioactivity. In this regard, it will be noted that in Fig. 2 the ascending curve of plasma activity is humplike in form. This hump indicates that plasma radioactivity approached equilibrium with the descending curve of ascitic radioactivity before the latter had reached the slow phase of metabolic decay. This finding was observed frequently and is not considered to be artefact. Although no definite interpretation is possible with the available data, it seems probable that the hump can be best explained on the basis of the above discussion and indicates equilibration of plasma and ascitic fluid albumin more rapidly than equilibration of plasma albumin and some other as yet unknown pool or pools of extravascular albumin.

The exchange rates between plasma and ascitic fluid as calculated with a single rate of transfer ranged between 0.40 and 0.65 Gm. per hour. Berson and co-workers[3] employing similar methods of analysis have reported in one patient an exchange rate of 0.8 Gm. per hour. It is noted than in F. H. the exchange rate decreased 60 per cent over a period of eight and one-half months during the course of improvement and spontaneous diminution of ascites. In S. G., who did not respond to therapy and whose ascitic volume increased, no changes in exchange rate were observed in serial studies three months apart. In a previous communication,[1] we have attempted to relate alterations in albumin exchange rates to changes in capillary permeability. The lack of consideration of extravascular pools other than the peritoneal cavity and the inability to sample fluid at membrane sites would seem to deter such interpretation with the present method of analysis. In addition, more data regarding the surface area of the membrane and pressure changes across the membrane will be required to establish the relationship of observed changes in albumin exchange rates to possible alterations in physical properties of the endothelial membrane.

SUMMARY

The concentration of radioactivity in the plasma and ascitic fluid following intravenous or intraperitoneal injection of albumin I^{131} was studied in both control subjects and in patients with Laennec's cirrhosis. Graphic representation of the data and mathematical analysis of the curves made it possible to calculate the transfer rate of albumin in the plasma and ascitic fluid. Repetitive studies in patients with cirrhosis during treatment disclosed a significant decrease in exchange rate in 1 case. In 3 control subjects and in 1 cirrhotic patient, 2 rates of distribution of injected radioactive albumin from the plasma into the extravascular compartment were observed. The data suggested that extravascular albumin exists in several pools, each with a characteristic size and exchange rate.

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THE INCORPORATION OF N^{15}-LABELED GLYCINE IN THE PLASMA PROTEIN, PLASMA PHOSPHOLIPIDS, BLOOD NONPROTEIN NITROGEN, AND SPLENIC TISSUE IN MAN

D. S. Amatuzio, M.D., M.S., P. S. Hagen, M.D., and R. L. Evans, Ph.D.*
Minneapolis, Minn.

A STATE of dynamic equilibrium with a constant exchange of nitrogen between various tissues has been accepted and supported with isotopic amino acid data. The incorporation of amino acids into plasma protein has been demonstrated with the use of C^{14} glycine, S^{35} methionine, and N^{15} glycine. This study extends the above in that plasma protein, plasma phospholipid, and nonprotein nitrogen were measured after the feeding of labeled glycine. Two men were given N^{15}-labeled glycine, and the plasma protein, plasma phospholipids, nonprotein nitrogen, and splenic tissue were measured for atom excess of N^{15}. The possible relationship between the circulating plasma protein nitrogen and body tissue protein nitrogen is discussed.

MATERIALS AND METHODS

Two patients with hereditary spherocytosis were fed N^{15}-labeled glycine, and the nitrogen of subsequent tissue samples was prepared for mass spectrographic isotope analysis.[1, 2]

Patient J. B., aged 73 years, weight 140 pounds, received 42.5 Gm. of 33 per cent atom excess of N^{15}-labeled glycine during 3 and ¾ days and had an estimated blood volume of 5,000 ml. The blood hemoglobin was 9.1 Gm. per 100 ml., and the plasma proteins 6.0 Gm. per 100 ml. Likewise Patient J. P., aged 65 years, weight 154 pounds, received 53 Gm. of 33 atom per cent excess of N^{15}-labeled glycin in 24 hours. He had an estimated blood volume of 5,400 ml. (1824-dye). The blood hemoglobin was 11 Gm. per 100 ml. and the plasma proteins 6.8 Gm. per 100 ml.

The N^{15}-labeled constituents of the plasma proteins, the plasma phospholipids, the blood nonprotein nitrogen, and splenic tissue were studied. The patients' weights remained the same throughout the experiment.

RESULTS

After the feeding of the N^{15}-labeled glycine to an adult, the isotope concentration in the total plasma proteins, the plasma phospholipids, and the nonprotein nitrogen were plotted against time (Figs. 1 and 2). Shortly after the feeding of the labeled glycine, the isotope concentration in the phospholipids, nonprotein nitrogen, and the blood plasma proteins rose rapidly to a maximum and then gradually declined (Figs. 1 and 2). Immediately after the feeding, the nonprotein nitrogen had an N^{15} atom per cent excess of three times that of

From the Department of Medicine, University of Minnesota and the Medical Service of the Veterans Hospital, Minneapolis, Minn.

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*At present a member of the Department of Physiology, University of Minnesota.

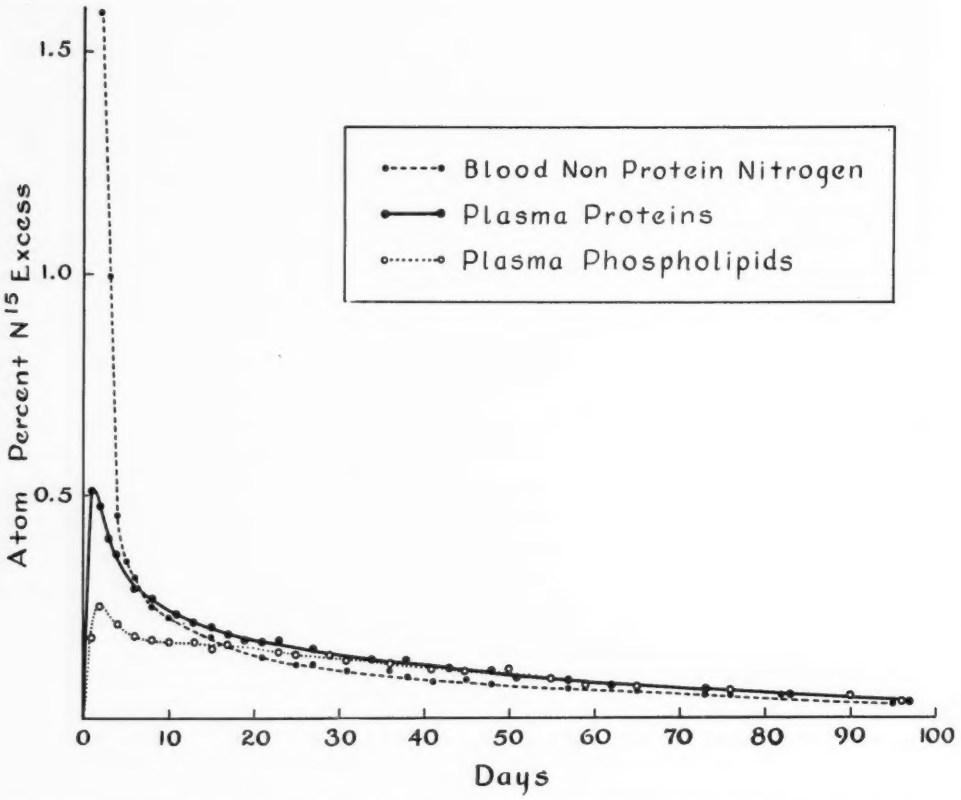

Fig. 1.—Patient J. P. received 53 Gm. of 33 per cent atom excess of N^{15}-labeled glycine in 24 hours.

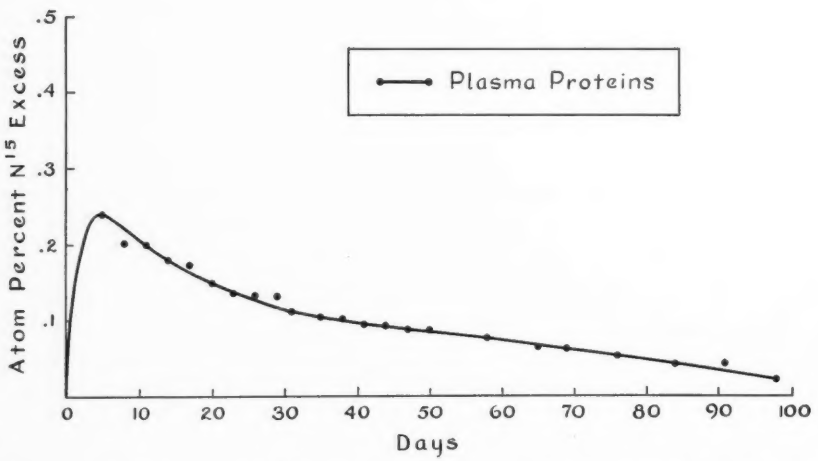

Fig. 2.—Patient J. B. received 42.5 Gm. of 33 per cent atom excess of N^{15}-labeled glycine in 3 and ¾ days.

the plasma proteins. The curves of N^{15} excess for Patient J. P. of the plasma proteins, plasma phospholipids, and the nonprotein nitrogen (Fig. 1) tended to approach the same isotope concentration at approximately day 30, and thereafter.

The curve of atom N^{15} excess for patient J. B. (Fig. 2) was similar to the above and a splenectomy was performed at day 69. The splenic pulp (devoid of red blood cells and plasma proteins) had the same atom per cent N^{15} excess as the plasma protein sample taken at day 69, being 0.066 and 0.062, respectively.

The relationship between the circulating N^{15}-labeled plasma protein and tissue utilization is complex, since it is a reflection of the metabolic activity of many tissues. Another complicating factor is that these tissues all have inde-

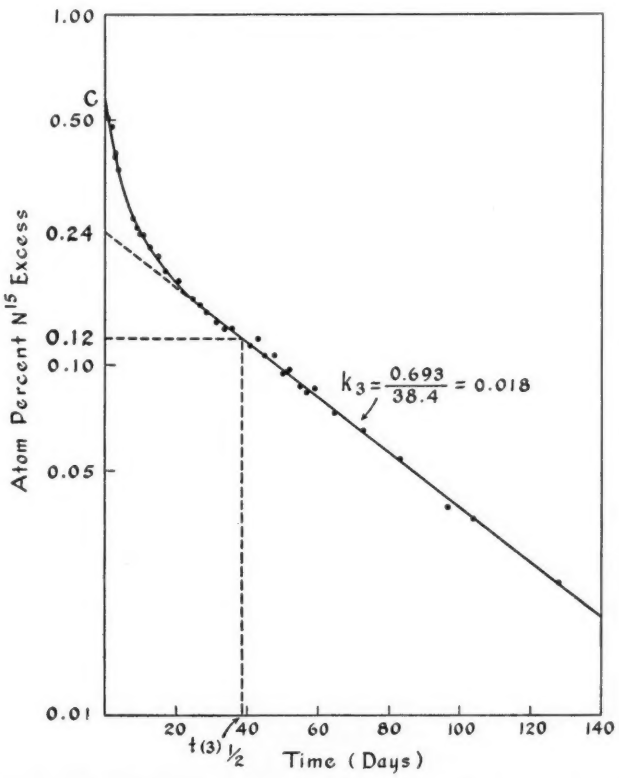

Fig. 3.—A graphic subtraction method to express and illustrate the separation of the first exponential component of a three-component exponential curve for Patient J. P.

pendent rates of protein utilization. When and if there are a number of such simultaneous parallel processes occurring independently of each other, the net change is the sum of the independent reactions. This total change is represented by the disappearance curve of the atom per cent N^{15} excess of the plasma protein.*

*An alternative theoretical scheme of Protein Metabolism and Interactions can be found in Science **118**: 558, 1953 (Evans, R. L., and Amatuzio, D. S.).

After a mixing time, the disappearance curve of the labeled plasma protein (Figs. 1 and 2) is not a simple semilogarithmic function of time but apparently changes with time to a multiphasic curve. The actual disappearance curve is represented empirically by the following general equation.

$$C = Ae^{-k_1 t} + Be^{-k_2 t} + De^{-k_3 t} + \ldots . \quad (1)$$

On using the above assumption of simultaneous, parallel, and independent processes, we break up the net equation above into its components:

$$\begin{aligned} C_1 &= Ae^{-k_1 t} \\ C_2 &= Be^{-k_2 t} \\ C_3 &= De^{-k_3 t} \end{aligned} \quad (2)$$

and normalize our units by choosing their magnitude such as to make

$$\text{initial } C = A + B + D + \ldots . \quad (3)$$

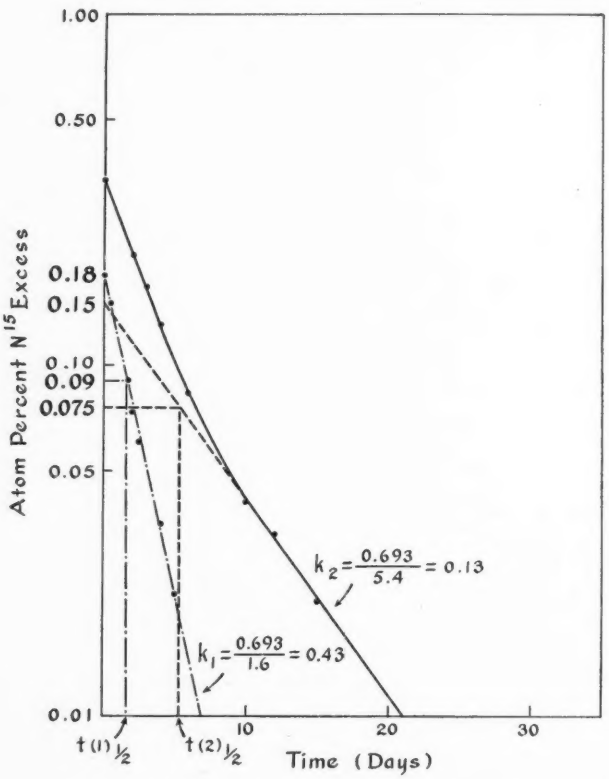

Fig. 4.—Continuation of the graphic subtraction of Fig. 3.

For the separation and specific evaluation of each distinct component, a graphic method is used. By the plotting of the logarithm of an observed concentration against time, t, a separation of the individual components in the disappearance curve can be done by the successive subtracting of the longest-lasting term in a sort of "peeling off" process. For the particular case of the plasma protein curve of Fig. 1, the first step of this procedure is shown in Fig.

3 the experimental points and sketched curve are shown on semilogarithmic graph paper, together with the straight line representing the longest-lasting component

$$De^{-k_3t} = \frac{0.24}{0.57} e^{-0.018t} = 0.42e^{-0.018t}.$$

The value 0.24 is the intercept at $t = 0$ of the straight line, the normalizing factor 1/0.57 is taken from the intercept at $t = 0$ of the curve, and 0.018 is 0.693 (or $\log_e 2$) times the reciprocal of the half-time, 38.4 days. This half-time is the time in which the amount of the particular component is reduced by a factor of 2.

In the next step of the procedure, one plots (the observed concentration – the already-obtained slowest component) on a similar semilog plot and repeats the previous step with this second graph, as shown in Fig. 4 where the time scale has been magnified for improved accuracy. In this way it is found that

$$Be^{-k_2t} = \frac{0.15}{0.57} e^{-0.13t} = 0.26e^{-0.13t}.$$

On continuing in similar fashion, one finds as a final approximation

$$Ae^{-k_1t} = \frac{0.18}{0.57} e^{-0.43t} = 0.32e^{-0.43t}.$$

As a final result then, equation (*1*) becomes

$$C = 0.32e^{-0.43t} + 0.26e^{-0.13t} + 0.42e^{-0.018t}. \quad (4)$$

for the plasma protein curve of Fig. 1.

On the applying of the above method to the curve of Fig. 2, it is found that for that case

$$C = 0.24e^{-0.10t} + 0.76e^{-0.018t}. \quad (5)$$

This case may lack the shortest-lasting term because of greater time delay in the collecting of the blood samples.

Similar treatment of the N^{15}-labeled plasma phospholipid curve of Fig. 1 yields the equation

$$C = 0.31e^{-0.36t} + 0.69e^{-0.016t} \quad (6)$$

for the plasma phospholipid.

DISCUSSION

In protein metabolism a dynamic state was first proposed by Borsook[3] and supported by Madden and Whipple,[4] and Schoenheimer and co-workers.[5] By the use of isotopically-labeled amino acids, Schoenheimer and co-workers showed that the body constituents were in a continuous state of interchange, synthesis, and degradation.[6] The present prolonged disappearance curves with estimated disappearance rates (Figs. 1 and 2) apparently measured substrate N^{15} reutilization and degradation with a gradual elimination of N^{15}-labeled constituents from the body. The initial rapid disappearance rate of 43 and 13 per cent per day may represent the body distribution and initial tissue uptake of the labeled constituent, and the slow disappearance rate of 1.8 per cent per day may be indicative of body conservation of nitrogen due to the continuous interchange,

synthesis, and degradation of nitrogen constituents. From the present study, to assign such values to the disappearance of the atom per cent N^{15} excess as measured in the plasma proteins and plasma phospholipids is arbitrary. It is not possible to assign a turnover time to any specific blood constituent, such as plasma protein or the plasma phospholipid, because the nitrogen atom enters into many complex metabolic systems of the body.

It has been demonstrated from previous studies that plasma proteins are gradually removed and utilized by cellular tissue.[7, 8] The present study is in agreement, where the N^{15} atom excess of splenic tissue (devoid of plasma protein and red blood cells) and the plasma proteins were similar in N^{15} atom excess content at day 69 for Patient J. B. The observed N^{15}-labeled plasma protein curve would then be assumed to reflect cellular uptake and utilization and would be representative of a net change of many independent simultaneous tissue rates of the body.

Since the blood nonprotein nitrogen arises from protein metabolism, the N^{15}-labeled nonprotein nitrogen disappearance curve should be an index of labeled protein degradation. The disappearance curves (Fig. 1) for plasma protein and nonprotein nitrogen are parallel and indicate an association where the labeled N^{15} nonprotein nitrogen may arise indirectly from the labeled N^{15} plasma protein with subsequent loss from the system. This is consistent with previous studies where a continuous loss of the isotope in man through body metabolim occurred with isotope excretion in the urine, feces, and/or breath,[9, 10, 11] and where, in mammalian experiments, a gradual disappearance of the isotope concentration was found[13, 14, 15] in each of the organs measured.

SUMMARY

From the present type of study only a general consideration of the labeled constituent can be made. The feeding of a substrate labeled with a universal atom of the body, such as isotopic C, N, S, or H, creates a complex system since these atoms enter into many metabolic pathways of the body.

The disappearance rates of the atom per cent N^{15} excess in plasma protein were approximately 43, 13, and 1.8 per cent per day. The fast rate of disappearance may represent body distribution and the initial tissue absorption, while the slower rate may represent body conservation of nitrogen. The disappearance rate of the atom per cent excess of nitrogen in plasma phospholipid was also of a similar magnitude.

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ELECTROPHORETIC STUDY OF HUMAN URINARY PROTEIN IN DISEASE

ALVIN L. SELLERS, M.D., AND JESSIE MARMORSTON, M.D
LOS ANGELES, CALIF.

THE urine of normal human subjects may contain up to 100 mg. of protein in twenty-four hours. An increase in urinary protein excretion well above this level is one of the most characteristic signs of renal disease. It is this finding that commonly brings patients with renal disease to the attention of the practicing physician. Thus, in all stages of glomerular nephritis, pyelonephritis, and nephrotic syndrome, increased proteinuria is the rule. Many other disease states, including toxemia of pregnancy, diabetes mellitus, disseminated lupus erythematosus, multiple myeloma, arterial hypertension, and congestive heart failure, are frequently associated with increased urinary protein excretion.

The urinary proteins excreted by normal man have been described by Rigas and Heller,[1] and more recently by McGarry, Sehon, and Rose.[2] They have shown that the small amount of protein in normal urine consists of albumin and globulins, but the ratio of these two classes of protein is the reverse of that found in normal plasma. There have been numerous reports on electrophoretic studies of the urinary proteins in the nephrotic syndrome[3-5] and multiple myeloma[6] but only scattered observations on the proteins excreted in various of the other diseases listed above.

In the present report, the urinary proteins excreted in all stages of glomerular nephritis are re-examined by electrophoretic methods, and those excreted in a variety of other disorders characterized by increased proteinuria are described.

METHODS

Preparation of Urine.—Voided urine was collected from 8:00 P.M. through 8:00 A.M. the following morning. The volume was measured and the urine filtered through Whatman No. 4 paper. An aliquot was taken for protein determination. An amount of urine containing 200 to 250 mg. of protein was placed in a cellophane bag and dialyzed for at least twenty hours against running distilled water at 2° C. The dialyzed urine was then lyophilized, and the dried protein was dissolved in sufficient cold 0.1 M Veronal buffer, pH 8.6, to result in a final protein concentration of 20 mg. per milliliter. This solution was dialyzed against 0.1 M Veronal buffer in the cold for at least twenty hours.

Electrophoretic Analysis.—All electrophoretic analyses were carried out at 0° C. in the Perkin-Elmer Tiselius apparatus as described previously.[7]

Protein Analysis.—Total urinary proteins were determined by a modification of the Kingsley biuret method.[8]

From the Institute for Medical Research, Cedars of Lebanon Hospital, and the Department of Medicine, University of Southern California, School of Medicine, Los Angeles, Calif.

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RESULTS

Urinary proteins were identified as albumin or a particular globulin by virtue of their electric mobility and their characteristic position in the electrophoretic pattern as compared with patterns of normal human plasma proteins. It should be understood that the name given a particular urinary protein fraction indicates that the fraction is electrophoretically similar to its analogue in plasma.

The per cent composition of the urinary proteins and, in nearly all cases, the electric mobility of the individual protein fractions are given in Table I.

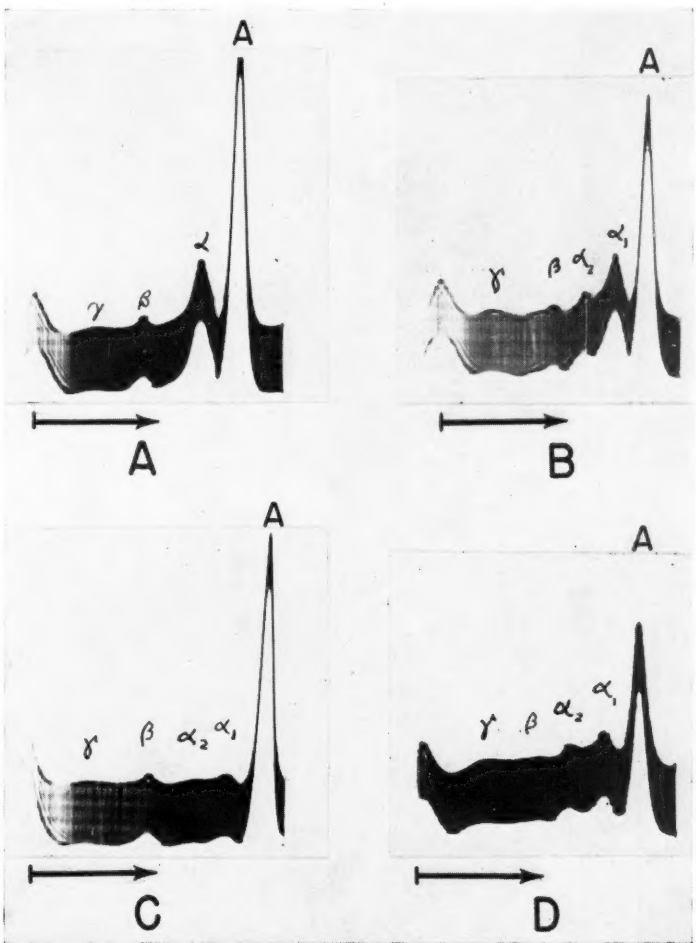

Fig. 1.—Electrophoretic patterns of urinary proteins in disease: *A*, malignant hypertension (F. J.); *B*, non-malignant essential hypertension (F. S.); *C*, chronic pyelonephritis (D. B.); *D*, acute renal failure (S. K.).

It is apparent that the degree of variation in the per cent composition of the urinary proteins in the various diseases studied will not allow quantitative statements to be made based on the number of patients reported here.

TABLE I. PER CENT COMPOSITION AND ELECTRIC MOBILITY OF URINARY PROTEINS

PATIENT	PER CENT COMPOSITION						MOBILITY (1×10^5 CM.^{2}/VOLT/SEC.)				
	ALBUMIN	GLOBULIN				PROTEIN (TOTAL MG./ML.)	ALBUMIN	GLOBULIN			
		ALPHA 1	ALPHA 2	BETA	GAMMA			ALPHA 1	ALPHA 2	BETA	GAMMA
Malignant Hypertension											
Mc G.	81.4	6.7	6.1	4.5	1.3	4+					
F. J.	65.3	21.8	3.6	5.2	4.0	7.1	5.61	4.42	3.58	2.81	1.83
M. J.	47.6	17.7	11.2	13.7	9.7	1.46	5.96	4.75	4.09	2.87	1.58
Nonmalignant Essential Hypertension											
F. S.	56.3	12.7	7.6	13.1	11.3	0.54	5.99	4.50		2.93	1.10
A. R.	64.3	25.7	2.1	4.1	3.8	5.92	5.93	4.90	4.06	2.96	1.25
V. A.	59.0	22.7	8.3	6.0	3.9	8.0	5.74	4.62	3.79	2.98	1.16
Chronic Glomerular Nephritis											
D. W.	85.8	3.4	3.4	4.8	2.5	1.9	6.49	5.47		3.58	2.22
W. S.	61.8	15.5	7.9	10.6	4.3	1.7	6.23	5.20	4.19	3.16	2.07
F. A.	77.7	9.2	6.9	3.6	2.6	2.5	6.24		4.12	2.92	2.12
R. S.	69.5	8.2	7.0	8.9	6.6	4+	5.61	4.53	3.87	2.58	0.96
L. G.	75.4	1.1	8.4	7.2	8.0	1.1	6.02	5.16		2.89	1.35
Chronic Pyelonephritis											
D. B.	74.7	5.7	5.1	8.2	6.3	1.78	6.28	4.89	4.12	2.94	1.37
S. G.	68.5	15.2	4.4	4.4	7.5	–	6.16	5.01	4.12	3.15	1.56
C. B.	72.9	5.5	4.4	8.5	8.8	3.5	5.98	4.75	4.06	2.84	1.38
Nephrotic Syndrome											
W. D.	61.5	15.4	16.3	6.8	–	10.0					
S. P.	75.0	9.1	10.8	5.1	–	9.5					
S. C.	69.3	–	12.1	14.0	4.7	3.28	5.84		4.10	2.65	1.21
I. H.	55.0	18.6	12.9	6.9	6.6	9.94	5.97	4.85	3.89	2.83	1.24

					Acute Renal Failure						
M. D.	49.3	23.3	6.8	8.9	11.7	7.8	6.04	4.81		3.04	1.81
S. K.	46.3	18.1	12.2	12.2	11.2	1.87	5.92	4.73	3.76	2.30	1.38
E. G.	45.8	5.1	17.0	22.6	9.4	–	6.72	5.51	4.18		1.77
					Disseminated Lupus Erythematosus						
L. H.	75.7	13.7	1.6	3.2	5.8	3.33					
F. C.	66.9	12.2		6.7	14.2	1.9	6.21	4.76		3.06	1.75
F. C.	66.5	11.2		8.1	14.2	0.61					
					Diabetic Glomerulosclerosis						
J. U.	86.3	6.9	3.6	3.3	–	2.0					
D. E.	85.0	7.9	2.5	7.6	–	8.5					
P. A.	88.1	4.2	3.1	4.6	–	1.0					
N. L.	70.1	16.5		9.1	4.3	5.54	5.50	4.33		3.23	1.90
P. R.	50.2	21.9	8.3	10.9	8.7	4.62	6.28	4.75	3.88	2.76	0.81
					Toxemia of Pregnancy						
C. R.	57.6	24.3	10.2	8.0	–	7.16					
M. H.	72.7	14.7		10.4	2.3	0.86	6.10	4.87		3.10	1.62
E. G.	56.8	17.0		12.4	13.8	2.46	5.72	4.60		2.76	1.49
					Multiple Myeloma						
P. C.		13.4		85.2		1.52			4.26	*2.40*	
T. P.	6.5	12.4		81.2		2.3	5.68	4.47		*2.44*	
T. K.	3.7	7.1		88.3	0.9	1.92	6.72	4.80		*2.59*	1.48

In the majority of instances in which proteinuria is increased, albumin, and alpha, beta, and gamma globulin are present. Except in patients with acute renal failure and multiple myeloma, the albumin fraction is greater than the combined globulin fractions.

The predominant urinary protein in multiple myeloma is, of course, a compound with an electric mobility similar to plasma beta globulin (Bence Jones protein). In addition, however, one can often demonstrate albumin, alpha globulin, and even small amounts of gamma globulin.

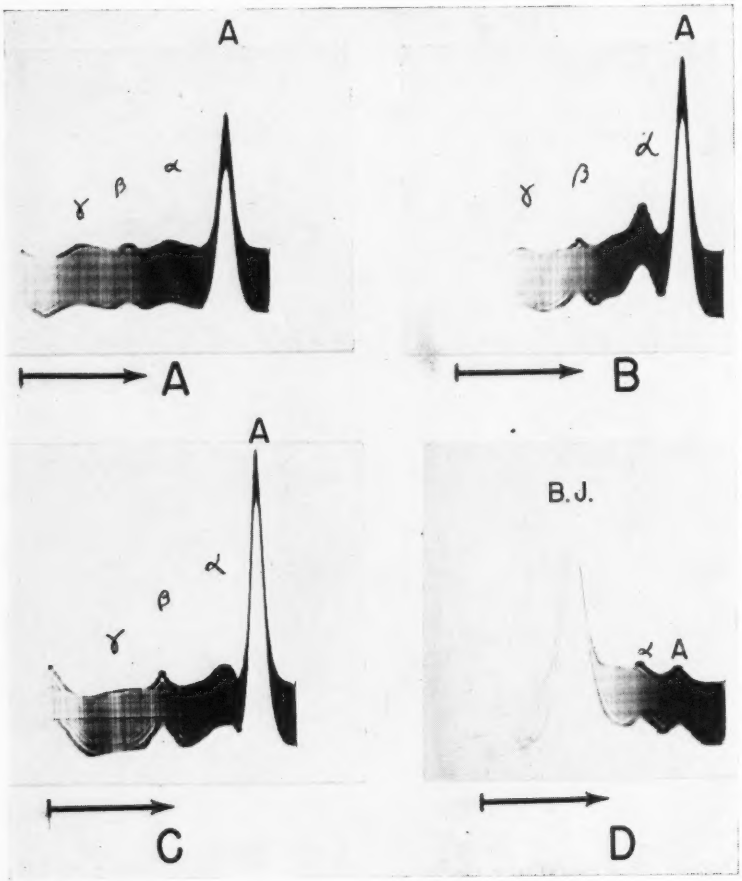

Fig. 2.—Electrophoretic patterns of urinary proteins in disease: *A*, disseminated lupus erythematosus (F. C.); *B*, diabetic glomerulosclerosis (P. R.); *C*, toxemia of pregnancy (M. H.); *D*, multiple myeloma (S. P.).

Urinary proteins from a total of 23 patients were examined. Electrophoretic patterns of some of these urinary proteins are shown in Figs. 1 and 2.

DISCUSSION

It was our hope that a survey of the urinary proteins excreted in disease might reveal patterns that were characteristic of one condition or another and thus prove useful in diagnosis. Our data do not allow quantitative statements,

but, with the exception of the excretion of Bence Jones protein in the urine of patients with multiple myeloma, there is no characteristic pattern of urinary proteins of any of the diseases studied. The variation in per cent composition of the urinary proteins between individuals in a single disease group is so marked that one cannot distinguish one disease state from another when confronted with only the electrophoresis pattern of the excreted proteins. Where it has been possible to gather electrophoresis data of urinary proteins from the literature, we find that in general our results are in agreement. Some published data from patients with nephrotic syndrome are shown in Table II. The conditions under which these several studies were made are not precisely the same. Nevertheless, the wide variation from patient to patient is clearly seen. Leutscher's data[5] on chronic glomerular nephritis are similar to our own.

TABLE II. PER CENT COMPOSITION OF URINARY PROTEINS IN DISEASE

AUTHORS	DISEASE	ALBUMIN	GLOBULIN ALPHA	GLOBULIN BETA	GLOBULIN GAMMA	TOTAL URINARY PROTEIN (GM. PER 100 ML.)
Longsworth and MacInnes[3]	Nephrosis	83.7	3.87	6.97	4.65	1.29
		67.6	9.4	10.8	12.2	1.39
Blackman and Davis[4]	Nephrosis	62.5-69.0	4.8- 9.2	8.9-11.2	16.0-19.4	0.47-0.76
		61.5-62.3	3.4- 4.5	9.4-12.0	22.0-24.8	1.17-0.67
		69.1-75.8	8.3-10.0	8.7-11.5	7.2-10.3	0.45-0.60
Luetscher[5]	Nephrosis	92.0	1.7	5.3	1.0	1.0
		83.3	4.5	10.4	1.8	0.6
		67.7	5.9	15.2	11.2	0.4
Luetscher[5]	Chr. glomerular nephritis	68.7	3.4	14.7	13.2	0.2
		80.5	3.1	8.3	8.1	0.15
Gutman et al.[6]	Multiple myeloma	5.0		95		0.33
		24.0		76		0.91
		0		100		0.28
		0		100		1.41
		8.0		92.0		0.42
		8.0		92.0		0.61
Rifkin and Peterman[10]	Diabetic glomerular sclerosis	52	22	8.9	11.3	1.9
		65	10	9	8.5	0.8
		74	9	5.6	5.8	0.9

The only report available to us on electrophoretic studies of urinary protein in toxemia of late pregnancy is that of Parviainen, Soiva, and Ehrnrooth.[9] They reported cases of mild and severe pre-eclampsia and eclampsia. There is no clear difference in per cent composition of the urinary proteins in these three groups. Albumin varied from 30.4 to 67.2 per cent, alpha globulin from 11.4 to 28 per cent, beta globulin from 9.1 to 16.5, and gamma globulin from 0 to 9.3 per cent.

Rifkin and Peterman[10] have performed electrophoretic analysis on urinary proteins in diabetic glomerulosclerosis. A great deal of variation in per cent composition of the urinary proteins exists among the three patients studied. Nevertheless their results are not unlike those reported in this paper.

It is well known that Bence Jones proteinuria is characteristic of multiple myeloma. The analyses of patients with multiple myeloma were included in this study in order to emphasize that proteins other than Bence Jones protein may appear in the urine in this disease.

We know of no analysis of the urinary proteins in benign or malignant hypertension, pyelonephritis, acute renal failure, or disseminated lupus erythematosus similar to those performed above.

SUMMARY

A survey of electrophoresis patterns of human urinary proteins in disease is presented.

In the disease states studied, with the exception of multiple myeloma, when proteinuria is increased, the predominant urinary protein has the electrophoretic characteristics of plasma albumin. The urinary excretion of plasma globulins is also increased.

Multiple myeloma excluded, the variation in distribution of urinary proteins among individuals of a given disease group is so great that patterns characteristic of one disease or another could not be distinguished with certainty.

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DETERMINATION OF THE BLOOD VOLUME USING RADIOACTIVE Cr^{51}: MODIFICATIONS OF THE ORIGINAL TECHNIQUE

WALTER J. SMALL, M.S., AND MARTINUS C. VERLOOP, M.D.
BOSTON, MASS.

UNTIL recently, estimations of the blood volume were almost always performed by determining the plasma volume and calculating the red cell mass and thus the total blood volume from the venous hematocrit. Evans blue dye (T-1824) has been most commonly used.[1] In the hands of well-trained individuals using a meticulous technique, this method gives good results but has certain disadvantages. Thus, hemolysis and lipemia may cause difficulty in the reading of the samples in the photoelectric colorimeter. Other possibilities for error exist, as pointed out by Reeve.[2] More recently, radioactive isotopes, particularly radioactive iodine in albumin, have been used to estimate plasma volume.[3] Total red cell volume can be calculated from the plasma volume and the venous hematocrit, assuming that the latter is the same as the body hematocrit. Recent studies by Gibson,[3] Gregerson,[4] and Edelman[5] indicate, however, that the normal hematocrit is approximately 0.9 of the venous hematocrit, although this ratio is by no means fixed. What is more, the degree of variation of this ratio in disease is not known.

Several methods have been used to determine the red cell mass. Both the carbon monoxide method and the Ashby method have been largely replaced by techniques using radioactive isotopes such as radioactive iron or radioactive phosphorus. For studies using radioactive iron, donors are essential, which is rather inconvenient. As for P^{32}, this material becomes eluted from the red cells rather quickly and will then retag other red cells. According to Reeve,[2] the mean rate of loss of P^{32} during the first hour is about 6 per cent of the total amount injected. Thus the calculation of the red cell volume by this method becomes difficult.

In 1950 Gray and Sterling[6] described the ability of radioactive chromium in the form of $Na_2Cr^{51}O_4$ to tag red blood cells and the use of this technique to determine the red cell volume. In contrast to tagging with P^{32}, the Cr^{51}-tagged cells retain their activity without significant loss to the plasma for about twenty-four hours after injection.

In 1953 Frank and Gray[7] described the labeling of plasma proteins by another chromium compound, i.e., $Cr^{51}Cl_3$. By consecutive use of the two chro-

From the Ziskind Laboratories (Blood Research Laboratory) of the New England Center Hospital and the Department of Medicine, Tufts University School of Medicine.

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mium compounds, the red cell mass and the plasma volume could be measured consecutively, and by combination of the two results, the total blood volume could be obtained.

The present paper is concerned with observations using Cr^{51} to determine both red cell mass and plasma volume. In the course of these studies, a constant source of error was found with the use of the published technique and was finally traced to the adsorption of chromium to the glassware. This was corrected by the use of a nonradioactive chromium chloride carrier.

METHODS AND MATERIALS

A. *Materials.*—Chromium compounds $Na_2Cr^{51}O_4$ and $Cr^{51}Cl_3$ were obtained from the Abbott Laboratories as sterile, isotonic, nonpyrogenic solutions. The specific activity on the date of shipment was 1 to 2 mc. per milligram, 1.5 mc. per milligram being the usual requirement. This indicates that even one half-life later the amount of chromium in 90μc (the maximum total dose used by us) was about 0.1 mg., well below the toxic dose of chromium.[7]

Ordinarily, 30μc Cr^{51} was used as $Na_2Cr^{51}O_4$ for estimating the red cell volume and 60μc Cr^{51} as $Cr^{51}Cl_3$ for plasma volume. This gave a maximum total amount of 90μc of Cr^{51} in a single blood volume determination. With the use of the formula of Marinelli, Quimby, and Hine,[8] and with the assumption that x-radiation affects the blood stream only and γ-rays affect the whole body, a dose of 90μc of Cr^{51} to a 70 kg. person gives a total dose of 0.09 rep, with the formula $D = D\beta + D\gamma$.

The total allowable whole-body dose for humans in rep is 0.300 rep per week; the amounts used were well below this.

Scintillation Counter.—

B. The scintillation counter used was a Nancy Woods well-type counter with a thallium activated sodium iodine crystal. The scaler used was a Tracerlab S.C. 33 1,000 scaler. Volumes of 2 ml. or less were ordinarily counted, thus obviating the need for correcting for absorption (geometry).

C. *Red Cell Volume.*—Instead of using washed red cells tagged with Cr^{51} as $Na^2Cr^{51}O^4$, we used whole blood. It was apparent that the less one handled the erythrocytes, the less likelihood there was of hemolysis and consequent errors. For the same reason we used heparin as an anticoagulant and not an ACD mixture.

The final technique worked out was as follows: Approximately 30 ml. of blood was withdrawn from an antecubital vein into a sterile heparinized syringe and incubated with 30μc Cr^{51} as Na_2CrO_4 in a sterile glass bottle at room temperature for one hour. The bottle was occasionally inverted very carefully during that time. At the end of an hour, 70 to 90 per cent of the radioactive chromate present was bound to the red cells, leaving 10 to 30 per cent free in the plasma. The higher the hematocrit, the more Cr^{51} was bound to the red cells; in cases of polycythemia this was very striking. After injection of whole blood, this part of the chromate rapidly disappeared from the plasma. Theoretically the Cr^{51} as chromate in the plasma could tag red cells in vivo.[9] To prevent this, Read[10] has suggested the addition of a powerful reducing agent before injection. He obtained good results with the addition of 50 mg. of vitamin C. This procedure was followed in our cases, the ascorbic acid being added to the tagged blood after the incubation period.

A carefully measured amount of tagged whole blood was then reinjected into the patient intravenously, leaving approximately 8 ml. in the bottle. From this 8 ml., 1 ml. was immediately diluted 1:99 with distilled water to obtain a counting rate susceptible to accurate assay. If red cells were left undiluted in the bottle, further tagging took place, giving rise to an error in the calculation of the radioactivity present in the injected red cells. From another 2 ml., a hematocrit determination was performed in the usual way, a standard hematocrit tube being filled to the 100 mark and centrifuged for one-half hour at 3,000 r.p.m. The remaining blood was also centrifuged and the radioactivity per milliliter of plasma determined. The amount of injected activity bound to red cells was determined by

subtracting the amount of activity in the plasma from the total activity injected. The activity in the plasma was ascertained from the measured activity per milliliter of plasma and the hematocrit of the sample, which defined the number of milliliters of plasma injected.

One-half hour after injection, a blood sample was withdrawn from the other arm and again a whole blood sample was counted. With the use of the venous hematocrit, the counts present per 1 ml. of packed red cells were calculated. No correction for trapped plasma was made,[11] because it was felt that the errors in the injected sample and the withdrawn sample probably cancelled out each other. By dividing the number of counts present in the injected red blood cells by the counts present per milliliter of patient's packed red cells, assayed in the sample withdrawn one-half hour later, the red cell volume could be calculated.

D. *Plasma Volume.*—For the determination of the plasma volume, $Cr^{51}Cl_3$ was used. As pointed out by Frank and Gray,[7] trivalent Cr^{51} shows a marked affinity for the plasma proteins. No more than 2 per cent may be expected to be bound by the erythrocytes when injected intravenously in humans. With the use of the diluting principle, plasma volume may be measured by dividing the number of counts injected minus 2 per cent for radioactivity lost to erythrocytes by the number of counts present in 1 ml. of plasma immediately after mixing in the circulation.

Technique: In a sterile glass bottle, 30 ml. of saline was mixed with 50 to 60μc Cr^{51} as $CrCl_3$. All but 2 ml. was injected intravenously, with careful measurement of the number of milliliters injected. By changing the syringe used to withdraw the half-hour sample for the determination of the red cell volume, we were able to inject the $Cr^{51}Cl_3$ with the use of the same needle. After 5 minutes, 10 minutes, 15 minutes, and 20 minutes, samples from the other arm were withdrawn and plasma counts were done. The counts per milliliter of plasma were plotted against time on semilogarithmic paper, thus giving a straight line. The theoretical count per milliliter of plasma at zero time could be extrapolated. From this figure, the number of counts present per milliter of plasma in the sample withdrawn immediately before injection of the saline $Cr^{51}Cl_3$ mixture was subtracted. For calculation of the number of counts injected, 1 ml. of the Cr^{51} mixture was diluted 1:99 with distilled water in a glass bottle. After a counting of a 2 ml. specimen of this diluted Cr^{51} saline mixture, the number of counts could be determined. The plasma volume was then:

$$\frac{\text{Total counts injected} - 2 \text{ per cent}}{\text{Counts per milliliter of plasma at zero time present in patient's circulation.}}$$

E. *A Modification of the Original Technique: Use of Chromium Chloride Carrier.*—Plasma volume determinations as first performed by the above method were found to give unusually low results. The possibility was considered that there might be adsorption of chromium to the glass wall of the bottles. Various studies demonstrated that Cr^{51} was indeed adsorbed to the walls of the glass beakers used for the dilution of the injected samples. This could be prevented by the addition of 10 mg. of the chromium chloride carrier to the distilled water before the addition of the 1 ml. Cr^{51} sample. Less carrier appeared to be insufficient to saturate the adsorption sites of the glass; more carrier gave no better results.

TABLE I

	CHROMIUM CARRIER	COUNTS PER 5′ PER ML.
A	0	9,472
B	5 mg.	13,081
C	10 mg.	14,203
D	20 mg.	14,649

By comparison of the effect of siliconized bottles and plastic bags with glass as containers for the diluted sample, it appeared that less adsorption took place in these containers than in glass; however, a small degree of adsorption took place which could be prevented by adding chromium carrier before dilution.

TABLE II

	CONTAINER	CHROMIUM CARRIER	COUNTS PER 5′ PER ML.
A	Uncoated glass beaker	0	12,763
B	Uncoated glass beaker	10 mg.	15,738
C	Siliconized beaker	0	14,700
D	Siliconized beaker	10 mg.	15,768
E	Plastic bag	0	14,279

The question remained whether adsorption of Cr^{51} also took place in the bottles used for the undiluted $Cr^{51}Cl_3$ saline mixture used for injection. It appeared that some adsorption also took place within one-half hour and could only be prevented by the addition of a sufficient amount of chromium carrier.

TABLE III

	CONTAINER	CHROMIUM CARRIER	COUNTS PER 5′ PER ML.
A	Uncoated glass bottle	0	15,861
B	Uncoated glass bottle	10 mg.	18,723
C	Siliconized glass bottle	0	15,712
D	Siliconized glass bottle	10 mg.	18,554

The counts were made in 1:99 diluted samples.

In practice it was of course not possible to use such a large dose of toxic chromium in addition to the fluid to be injected; also it was thought to be unnecessary. The Cr^{51} saline mixture, however, must be made just before injection and the dilution of the injected sample for counting must be made immediately after withdrawing the fluid in the syringe.

Did adsorption of Cr^{51} also take place in the bottles used for incubating and diluting the $Na^2Cr^{51}O^4$ in red cell volume studies? Experiments demonstrated that this was not the case with $Na^2Cr^{51}O^4$. We could not show any adsorption in the glass wall of the incubating bottle nor in the walls of the diluting glass beaker.

TABLE IV

	CONTAINER	CHROMIUM CARRIER	COUNTS PER 5′ PER ML.
A	Uncoated glass beaker	0	9,664
B	Uncoated glass beaker	10 mg.	9,566

It appeared from these studies that $Cr^{51}Cl_3$ was adsorbed by the glass wall while $Na^2Cr^{51}O^4$ was not. It is therefore possible that glass has an affinity for cations while not affecting anionic forms.

RESULTS

Results of Blood Volume Determinations in 2 Normal Individuals and in 4 Patients.—

1. *V., normal* white man, aged 38 years, venous hematocrit 48 per cent, not corrected for trapped plasma; height, 6′2″; weight, 180 pounds.

Red cell mass	2,190 ml.
Plasma volume	2,725 ml.
Total blood volume	4,915 ml.

Body hematocrit 44 per cent = 0.92 of the venous hematocrit.

2. *De F., normal* white man, aged 56 years, venous hematocrit 49 per cent, not corrected for trapped plasma; height, 5′3″; weight, 154 pounds.

Red cell mass	2,160 ml.
Plasma volume	3,150 ml.
Total blood volume	5,310 ml.

Body hematocrit 41 per cent = 0.86 of the venous hematocrit.

3. M., white man, aged 45 years, venous hematocrit 52 per cent, not corrected for trapped plasma; height, 5′6½″; weight, 176¼ pounds.

Red cell mass	2,218 ml.
Plasma volume	2,860 ml.
Total blood volume	5,078 ml.

Body hematocrit 44 per cent = 0.84 of the venous hematocrit.

A diagnosis of "stress-erythrocytosis"[12] was made in this case.

4. C., white man, aged 45 years, venous hematocrit 58 per cent, not corrected for trapped plasma; height, 5′4″; weight, 145 pounds.

Red cell mass	2,395 ml.
Plasma volume	2,230 ml.
Total blood volume	4,625 ml.

Body hematocrit 52 per cent = 0.9 of the venous hematocrit.

A diagnosis of "stress-erythrocytosis"[12] was made.

5. S., white man, aged 54 years, venous hematocrit 71 per cent, not corrected for plasma trapping; height, 5′7½″; weight, 185½ pounds.

Red cell mass	4,125 ml.
Plasma volume	1,905 ml.
Total blood volume	6,030 ml.

Body hematocrit 68 per cent = 0.96 of the venous hematocrit.

The diagnosis was made of anoxemic erythrocytosis, as the result of an oxygenation failure in the lungs.

6. E., white woman, aged 64 years, venous hematocrit 77 per cent, uncorrected for trapped plasma; height, 5′4″; weight, 154 pounds.

Red cell mass	5,110 ml.
Plasma volume	2,380 ml.
Total blood volume	7,490 ml.

Body hematocrit 68 per cent =0.88 of the venous hematocrit.

Diagnosis: polycythemia vera.

SUMMARY

In the course of blood volume determinations using the Cr^{51} method, a constant source of error was found with the use of the published technique. This was finally traced to the adsorption of chromium to the glassware and could be corrected by the use of nonradioactive chromium chloride carrier.

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SERUM CHOLESTEROL REDUCTION IN PATIENTS BY THE ORAL ADMINISTRATION OF A BRAIN EXTRACT

RICHARD J. JONES, M.A., M.D.
CHICAGO, ILL.

UNTIL the recent introduction of soya sterols,[1, 2] rigid restriction of dietary fat and cholesterol has been the only means of reducing the level of serum cholesterol in the human. The effectiveness of this has often been disappointing in practice. Best and co-workers[3] have demonstrated a consistent reduction of serum cholesterol by the use of a sitosterol preparation in a small group of normal subjects. In the past three years we have been studying the hypocholesterolemic effect of a different agent and can now report on a similar reduction in serum cholesterol levels with the administration of a simple extract of mammalian brain, rich in cerebrosides.

A crude residue of mammalian brain, remaining after serial extractions with acetone and then with ether, has been shown to depress the serum cholesterol and mitigate atherosclerosis[4] in cholesterol-fed chicks. A preliminary trial[5] on the effectiveness of this same material in patients gave sufficient promise so that a more potent product was sought. Because of the work of Rosenheim and Webster,[6] it was presumed that the activity lay in the cerebroside content of the brain powder. Taking advantage of the solubility characteristics of cerebrosides, we obtained the fraction described below, which was more potent and proved to give more consistent effects in patients than the original crude material.

MATERIAL AND METHODS

This cerebroside fraction of beef brain was obtained by subjecting the powdered crude residue mentioned above to extraction with boiling ethanol. Cerebrosides are quite soluble in boiling ethanol but precipitate on cooling, the proportion of various constituents depending upon the exact temperature. By use of the material which left solution at 4.0° C. and above, a fluffy tan to cream-colored powder was obtained. This powder contained approximately 1.65 per cent phosphorus and 11 per cent reducing sugars. Thus, it contained approximately 45 per cent sphingomyelin and phospholipids and something less than 50 per cent glycosides. There was no protein material remaining in this preparation by the biuret test.

With the use of the cholesterol-fed cockerel as an assay animal,[7] 10 Gm. per day of the above fraction was capable of 40 per cent depression of the elevated serum cholesterol levels, whereas a like amount of the cruder preparation effected only a 25 per cent reduction. There was a faint "fishy" taste and odor to the preparation, but this was not objectionable to most people. The very light powder was not soluble in water but easily formed an emulsion or gel with water on standing. Patients were asked to take three rounded

From the University of Chicago, Division of Biological Sciences, Department of Medicine, Chicago, Ill.

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teaspoonfuls (i.e., 8 to 10 Gm.) at each mealtime and at bedtime, in water, orange juice, or other beverages as might suit their tastes.* The maximum dose taken was thus about 40 Gm. per day, though most patients probably took closer to 30 Gm. per day.

The patients selected for this study were all outpatients, under care in the University of Chicago Clinics, who had been observed for six months to many years both clinically and with regard to their serum lipid levels. All had coronary artery disease as manifested by the anginal syndrome or an electrocardiographically proved myocardial infarction. They had varying degrees of hypercholesterolemia, one patient presenting an essential hyperlipemia with xanthoma tuberosum and tendinosum.† All agreed to try the treatment, which they were told was only intended to influence their blood cholesterol, and no great pressure was applied or censure implied should they find the material unpalatable. Three women rejected the brain extract as nauseating after trying it in various beverages, and, in fact, only one woman did follow the program. The nine patients reported here all insisted that they took the material as directed, and these are the only subjects, except for one medical student to be mentioned later, who have followed this dosage for such a period of study. These patients were already on diets as low in fat, cholesterol, and calories as they were willing to follow. Some had lost weight significantly when first seen in the clinic, but throughout the one-year period of observation reported here, the body weight remained constant, never fluctuating by more than 1 kg. from the average weight. At the time of the study, each patient kept a diary of his habitual food intake for one week and was asked to adhere as closely as possible to this diet while he took the cerebroside fraction. Their cholesterol intakes varied from roughly 200 to 400 mg. per day, and fat intake was estimated to vary from 25 to 80 Gm. per day by the clinic nutritionist, Miss Olson.

Serial determinations for 1 year, 6 months before and 6 months after the experimental period, provided the control data on the various serum lipid levels. In six of these patients, serial determinations over one or two previous years gave us further confidence in the mean pretreatment values reported. Each patient was bled at weekly to bimonthly intervals, in the same fasting state, throughout the year of observation. The frequency of blood sampling was once a week for the duration of cerebroside fraction administration (4 to 8 weeks) and for 2 weeks before and after that period. Diabetes and hypothyroidism were carefully excluded in each case. At least once during the course of administration of the powder, a complete blood count and routine urinalysis were done on each patient.

The serum was analyzed in duplicate for cholesterol by a modified Liebermann-Burchard reaction; total lipids were analyzed gravimetrically and lipid phosphorus colorimetrically, after extraction by the Bloor technique.[9] Five milliliters of the same serum samples were diluted with NaCl solution and KBr to a density of 1.21, centrifuged at 30,000 r.p.m., in the Spinco 30.2 rotor for 13 hours, and the top fraction removed. This top fraction was then submitted to analytical ultracentrifugation after dilution to 2.0 ml. with NaCl-KBr solution of density 1.21. The procedure of Lewis and Page[10] was followed except for the use of this dilution factor, the performance of the analytical runs at 20° C. instead of 26° C., and the measurement of the areas involved at the intervals 0, 2, 10, 18, 26, and 34 minutes after reaching 58,264 r.p.m. The beta lipoprotein peak was still off the photographed frame in most instances and so had to be estimated by extrapolation. No correction was made for the Johnston-Ogston effect.[11]

RESULTS

The average serum lipid values before, during, and after administration of the cerebroside fraction can be seen for each of the nine patients in Table I. In Fig. 1, it can be seen that each patient showed a similar reduction in mean total serum cholesterol of 11 to 26 per cent from control levels, an aver-

*We gratefully acknowledge the generosity of Wilson Laboratories who, through Dr. David Klein, supplied us with this "cerebroside fraction" in quantity.

†Our experience agrees with that of Lever and co-workers[8] that xanthoma tuberosum and tendinosum can occur with hyperlipemia as well as hypercholesteremia. Before any dietary treatment, this patient presented a turbid serum with neutral fat of 1,540 mg. per cent.

age of 20 per cent for the group. It is fairly apparent from Fig. 1 that the milligrams per cent of cholesterol reduction is not strictly proportional to the initial level of serum cholesterol. One normal medical student (not included in the data here) with a serum cholesterol of 157 mg. per cent showed no response to the same material taken for a period of one month.

TABLE I. THE EFFECT OF A CEREBROSIDE FRACTION OF BRAIN ON THE SERUM LIPIDS OF NINE PATIENTS

PATIENT		TOTAL SERUM CHOLESTEROL			TOTAL LIPID			LIPID PHOSPHORUS		
		BEFORE	DURING	AFTER	BEFORE	DURING	AFTER	BEFORE	DURING	AFTER
M. B.	N*	7	4	10	7	4	10	7	4	10
	Mean	408	302	352	1,070	845	978	13.2	11.9	12.4
	Max.	456	320	420	1,268	883	1,765	14.1	12.8	14.1
	Min.	368	284	274	958	808	845	12.0	10.4	9.8
K. R.	N	5	6	10	5	6	10	5	6	9
	Mean	343	272	309	1,116	922	992	12.1	10.8	11.5
	Max.	367	311	339	1,235	1,038	980	13.7	11.9	13.9
	Min.	320	222	272	1,020	888	890	10.6	9.9	10.2
P. D.	N	4	7	9	4	7	9	4	6	9
	Mean	347	268	267	1,019	844	889	11.7	10.1	10.5
	Max.	412	279	293	1,153	922	993	12.4	10.5	11.4
	Min.	276	260	241	823	775	828	10.5	9.9	9.5
S. B.	N	9	5	4	8	5	4	9	5	4
	Mean	345	281	307	1,242	1,195	1,194	13.0	11.4	12.2
	Max.	403	302	350	1,443	1,400	1,305	14.3	12.1	13.3
	Min.	289	263	283	915	1,083	1,135	12.0	10.2	11.3
H. K.	N	7	4	6	7	4	6	7	4	6
	Mean	308	229	284	900	762	872	11.4	9.9	10.6
	Max.	364	270	318	1,055	820	983	12.4	10.9	11.7
	Min.	261	193	262	813	718	803	10.8	9.1	9.5
R. G.	N	12	4	9	11	4	9	12	4	9
	Mean	333	295	322	926	822	1,000	10.7	10.0	11.3
	Max.	385	315	378	1,028	870	1,083	11.7	10.8	12.8
	Min.	315	276	281	828	783	923	9.4	9.0	9.8
C. A.	N	5	4	10	5	4	9	5	4	10
	Mean	270	215	240	844	652	774	9.8	8.6	9.9
	Max.	311	223	269	970	713	885	10.2	9.4	14.9
	Min.	215	207	207	753	615	690	9.4	6.8	8.3
M. M.	N	11	8	10	11	8	10	11	8	10
	Mean	269	230	245	829	727	770	9.7	8.8	9.8
	Max.	313	312	291	930	805	830	10.3	9.6	11.2
	Min.	244	195	202	750	601	703	9.1	8.1	7.8
P. S.	N	4	3	6	4	3	6	4	3	6
	Mean	270	209	268	824	709	894	9.6	9.1	10.7
	Max.	337	219	338	963	758	1,003	10.4	9.9	13.0
	Min.	215	201	203	710	645	793	8.8	8.3	9.3

*Number of Blood Samples.

From Table I it can be seen that the return to control (pretreatment) values is usually not complete. This is largely because there appears to be an inconstant residual effect, lasting for two to six weeks after stoppage of the material, which was not even apparent in five of the patients. However, this does not altogether explain the generally lower values of two patients who were followed for six and twelve months, respectively, and whose mean lipid levels were

appreciably below prior levels. This, we suspect, exemplifies the unpredictability of this disorder. Furthermore, we recognize that the total cholesterol, total lipid, and phospholipid determinations show increasing individual variation, in that order, in these patients with hypercholesterolemia of athero-

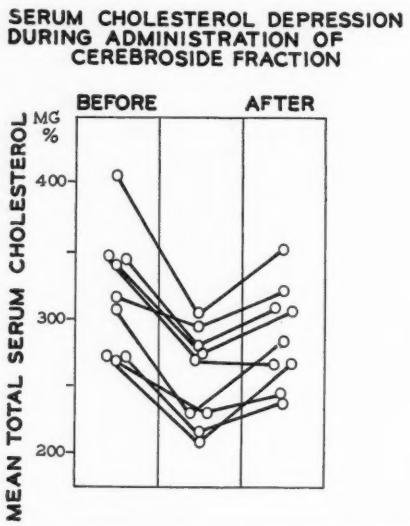

Fig. 1.—Each point represents the mean value for serum cholesterol, representing all determinations before, during, or after the period of cerebroside administration, for each patient. The number of determinations constituting each mean can be seen in Table I.

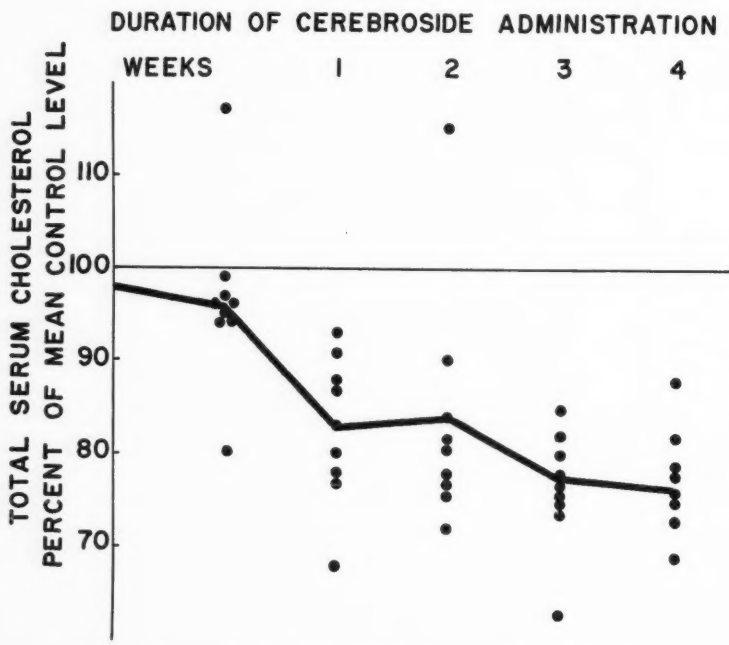

Fig. 2.—Individual values of total serum cholesterol expressed as per cent of mean control level immediately before and at weekly intervals during the first four weeks of cerebroside administration in each of the nine patients.

sclerosis.[12] However, not only the mean but usually the maximum and minimum values of each of the serum lipid moieties were also lowest while on the brain extract. Whether comparison is made with the control values before or after the experimental period, the average depression of total lipid, cholesterol, or phospholipid during that period is statistically highly significant ($P<0.01$). Inasmuch as the range of cholesterol values during pretreatment and treatment periods indicates virtually no overlapping in all but two of the patients, this is evident on inspection. In six cases the minimum cholesterol value varied from the lowest control value by 40 to 95 mg. per cent (Table I).

In Fig. 2 the consistency of the hypocholesteremic response can be seen. With one exception, all serum cholesterol values were well below the mean pretreatment levels and during the third and fourth weeks of administration fell to 23 per cent below the mean control level. The return to control levels was not graphed because the period of administration varied. In the longer periods of administration it was clear that, although there was still some fluctuation about the new mean level, the mean level remained definitely lower while on the extract than otherwise.

The phospholipid concentration of the serum was reduced but to a lesser extent, both percentagewise and absolutely, than the serum cholesterol (Fig. 3). Phospholipid concentration of the serum was reduced about half as much in milligrams per cent as the total cholesterol. Consequently, there was a reduction in the cholesterol: phospholipid ratio during ingestion of the cerebroside fraction. From this we might have anticipated a reduction in beta rather than alpha lipoprotein.

The total lipids were reduced during the experimental period by about 15 per cent, on the average. Each case showed a fall in total serum lipid (Fig. 3), though the amount was negligible in one patient (S. B.) Deducting total cholesterol and phospholipid from the total lipid concentrations to obtain neutral fat, we found an average reduction in neutral fat in all but this case. In Table II we see that the patient (S. B.) who had essential hyperlipemia with xanthoma tuberosum and tendinosum actually had an increase of 10 per cent in neutral fat as the cholesterol fell in the serum. The absolute reduction in concentration of neutral fat, in milligrams per cent, was less than that of the cholesterol in about half of the cases, but the average reduction in neutral fat, excluding S. B., was only 15 per cent.

TABLE II. THE INFLUENCE OF THE CEREBROSIDE FRACTION ON MEAN SERUM NEUTRAL FAT IN NINE PATIENTS

PATIENT	PRETREATMENT PERIOD (MG. %)	EXPERIMENTAL PERIOD (MG. %)	DIFFERENCE (MG. %)
M. B.	332	271	61
K. R.	471	381	90
P. D.	379	323	56
S. B.	572	629	–57
H. K.	307	286	21
R. G.	325	277	48
C. A.	329	223	107
M. M.	317	284	41
P. S.	302	272	30

The ultracentrifugal studies, summarized in Fig. 3, revealed no significant change, or perhaps a tendency to rise, in mean alpha lipoprotein, while, with one exception, there was a fall in beta lipoprotein during the experimental period. The latter change is of the same order of magnitude as that noted in the total lipid figures and amounts to about 17 per cent of the control level.

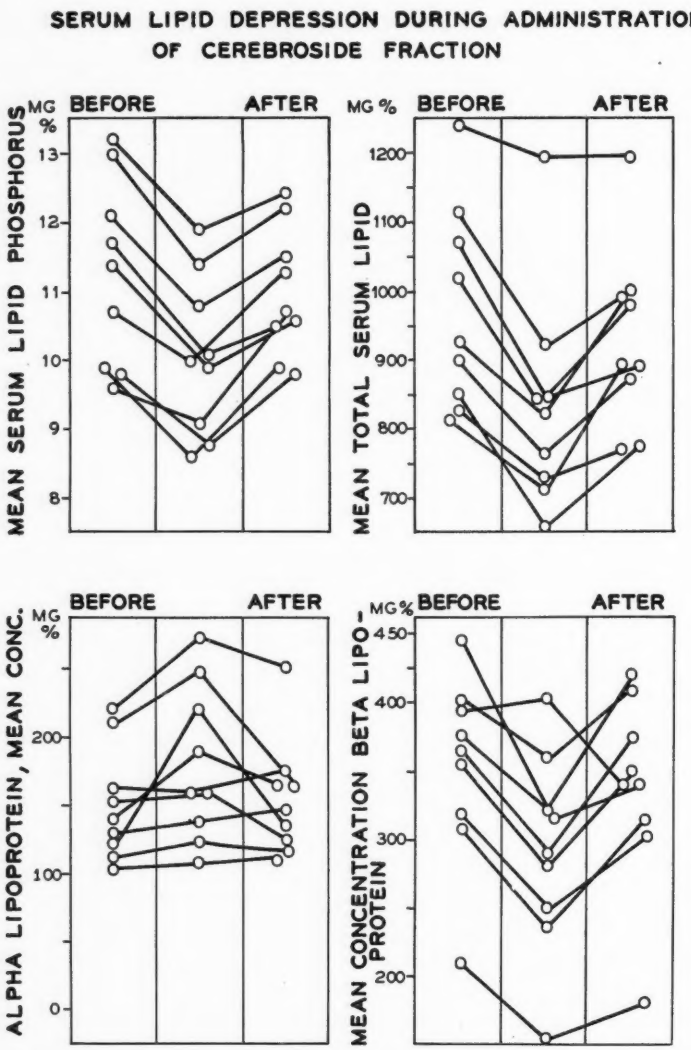

Fig. 3.—Mean values of serum lipid phosphorus, total serum lipid, beta lipoprotein ($-S_{1.21}$ 20-40), and alpha lipoprotein ($-S_{1.21}$ 0-10) in milligrams per cent in the nine patients displayed as in Fig. 1.

The only patient who had an increase in beta lipoprotein during the period of therapy was P.D., who had recently recovered from an attack of acute podagra. Changes in the $-S_{1.21}$ 40 to 70 range (Gofman's S_f 12-20) were in the same direction, though of dubious significance in three of the patients. There was

only one patient (S. B.) with a significant elevation of the $-S_{1.21}$ 100-400, and this did fall during therapy, though wide fluctuations between individual observations render the finding insignificant.

CLINICAL OBSERVATIONS

Aside from the nine patients mentioned above, three women who were invited to take the cerebroside fraction rejected it as causing nausea. The cooperating patients complained during the first week of increased borborygmi and flatus. Four of the nine complained of constipation. On one this was severe and required mineral oil for relief; but in two the constipation subsided after one week and in one it yielded to a more regular and soft movement than he had known for several years.

Six of these patients had been suffering with angina of effort. One had increased angina related to his constipation, and two felt some subjective improvement with therapy, though on more detailed questioning this was not convincing. There was no change noted in blood pressure or in routine urinalysis, hemoglobin determination, or leukocyte count. Three of the patients were on ambulatory Dicumarol therapy throughout the period of observation, and no coincident change in Dicumarol requirement was noted.

One patient (P. D.) developed podagra without hyperuricemia a few weeks before the initiation of the experimental period; this may have been gout and perhaps explains the great discrepancy in pre- and posttreatment levels, for there is some evidence that active gout influences serum lipid levels.[13] Another patient (P. S.) suffered an episode of crushing substernal pain precipitated by a protracted fit of anger, just as he was entering the fourth week on the cerebroside fraction. Serial electrocardiograms established the diagnosis of subendocardial infarction, presumably without coronary thrombosis or occlusion. For this reason his experimental period was terminated after only three weeks.

DISCUSSION

In view of the possible relationship between serum cholesterol level and certain diseases, notably atherosclerosis, it would be of great theoretic interest to be able to control the serum cholesterol level. It has been shown in animals[1] and in humans[2, 3] that a mixture of soya sterols, predominately sitosterol, will significantly lower the serum cholesterol. There is some evidence that the sitosterol competes for fatty acid esterification with cholesterol, intestinal absorption of cholesterol ester being particularly depressed.[14] Dihydrocholesterol has also been shown to be an effective inhibitor of cholesterol absorption in the cholesterol-fed chick.[15] Such substances as ferric chloride,[16] ethionine,[17] and a host of compounds related to cholesterol[18] have been shown to have a depressing effect on cholesterol metabolism in certain situations. So far, none of these latter compounds has been used in humans.

Rosenheim and Webster demonstrated that there was a factor in brain which was concentrated in the "white matter of Thudichum" and appeared to be the cerebroside phrenosin which, when fed to rats, caused conversion of up to 90 per cent of the fecal steroids into coprosterol.[6] Ordinarily, rat feces con-

tain only about 2 per cent coprosterol. Our original presumption was that in the feeding of brain matter to animals, the cholesterol pool of the body would constantly be depleted by conversion to the unabsorbable coprosterol of a portion of the intestinal cholesterol otherwise available for reabsorption. Recently, however, we have been unable to establish any change in coprosterol concentration in the feces of animals fed the cerebroside preparation used here, though the total fecal steroid excretion seems to be enhanced.

The results obtained by Best and associates[3] with soya sterol administration are certainly no more striking than those presented here. The fact that our mean hypocholesterolemic effect is somewhat more than theirs may be a consequence of the higher mean cholesterol level of our subjects. In general, the poorer response occurs in those subjects with the lowest cholesterol level, and there is probably a minimum level below which no response can be expected.

There remains some doubt that the primary effect in the serum is on the cholesterol level, perhaps through an influence on intestinal absorption, and that the reductions in neutral fat, phospholipid, and perhaps even the beta lipoprotein are all consequential. The cholesterol is more consistently reduced in this series and is most affected quantitatively. Neutral fat is probably not primarily affected, because in our case of essential hyperlipemia where there was a propensity to accumulate neutral fat in the serum, there was an actual rise in neutral fat even though cholesterol fell substantially. The phospholipid levels tended to parallel the cholesterol levels but not closely enough to maintain the original cholesterol:phospholipid ratio. The changes in beta lipoprotein, the alpha lipoprotein remaining constant, are compatible with this change in ratio. The cholesterol:phospholipid ratio in human beta lipoprotein is about 1.3:1.0 and in alpha lipoprotein 1:2.[19] Thus, a relative decrease in beta lipoprotein should cause a fall in the ratio as seen here.

SUMMARY

Nine subjects with coronary artery disease and varying degrees of hypercholesterolema were fed a cerebroside fraction of beef brain for periods of three to eight weeks. Each of the subjects showed a fall in serum cholesterol during the experimental period which was paralleled by significant but slightly lesser reduction in phospholipid, neutral fat, and beta lipoprotein. The alpha lipoprotein was not significantly affected.

This work could never have been accomplished without the excellent technical assistance of Sheldon Huffman, Irene Carr, Eugene Balter, and Henry Kallet. I am indebted to Dr. Lois Scheimann, Mr. Norman Penland, and Mrs. Helen Sosna for their technical assistance in performing the ultracentrifugal analyses. I am also grateful to Dr. O. K. Reiss for analysis of the cerebroside fraction and to Dr. Emmett Bay for comments on the manuscript.

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THE EFFECT OF PROTAMINE ON LIPEMIA

HUGO C. MOELLER, M.D., PH.D.,* LIONEL M. BERNSTEIN, M.D., PH.D.,
LUCILLE PALM, B.S., AND MORTON I. GROSSMAN, M.D., PH.D.
DENVER, COLO.

INTRODUCTION

A NUMBER of recent studies have demonstrated that intravenous injection of protamine causes an increase in lipid concentration in the blood.[6-11] No studies on human subjects and no studies on the effect of protamine on lipemia induced by intravenous injection of fat emulsion have been reported. The study reported here deals with these topics and related studies on the mechanism of action of protamine.

MATERIALS AND METHODS

The emulsion used in this study† had the following composition: Refined cottonseed oil 15 per cent, glucose 4 per cent, soybean phosphatide 1.2 per cent and Pluronic F-68 0.3 per cent. The protamine was in the form of a 1 per cent solution of protamine sulfate in physiologic saline.‡

Optical density, and free and total fatty acid concentration of serum samples were determined by methods previously described.[1] In those instances where analyses were performed on infranatant fractions of serum, these were obtained by high-speed centrifugation (14,000 r.p.m. for two 30-minute periods at 5° C.).

STUDIES IN HUMAN SUBJECTS

Intravenous Administration of Fat Emulsion.—Ten fasting normal male subjects received two separate 500 ml. infusions of fat emulsion one week apart. One of these infusions was given with and one without protamine sulfate. Half of the subjects received the protamine with the first infusion of fat emulsion, the other half with the second infusion. The intravenous infusion was started with 5 per cent dextrose in water, and the fat emulsion was run into the tubing of the dextrose infusion. The dextrose infusion was stopped when the fat emulsion was begun. The fat emulsion was given at 30 drops per minute for the first hour and 60 drops per minute thereafter until 500 ml. had been given.

The protamine sulfate was given as a separate intravenous infusion into a leg vein. Five hundred milligrams of protamine sulfate (10 mg. per milli-

From the U. S. Army Medical Nutrition Laboratory, Fitzsimons Army Hospital, Denver, Colo.

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*Captain, MC, U.S.A.R. Present address: Veterans Administration Research Hospital, 333 E. Huron St., Chicago, Ill.

†Lipomul, Research No. 10-867-3, Upjohn Company, Kalamazoo, Mich.

‡Eli Lilly & Company, Indianapolis, Ind.

liter) was mixed with 450 ml. of 0.9 per cent sodium chloride, giving a concentration of 1 mg. of protamine sulfate per milliliter. The subject received this mixture at 60 drops per minute (4 mg. per minute) for one-half hour immediately preceding the start of the fat infusion and at 30 drops per minute (2 mg. per minute) for the remainder of the infusion. The protamine and fat infusions ended at approximately the same time. The mean duration of the fat emulsion infusion was 220 minutes.

Ten venous blood samples were drawn in the following time sequence: (1) control; (2) 30 minutes after starting protamine, this sample coincided with the beginning of the fat infusion; (3) 60 minutes after start of fat infusion; (4) 120 minutes after start of fat infusion; (5) end of fat infusion; (6) 60 minutes after end of fat infusion; (7) 120 minutes after end of fat infusion; (8) 180 minutes after end of fat infusion; (9) 240 minutes after end of fat infusion; and (10) 300 minutes after end of fat infusion. Blood was drawn into chilled syringes and kept refrigerated (5° C.) during centrifugation and storage until analyses were performed.

Blood pressure, respiration rate, pulse rate, and rectal temperatures were recorded before and at intervals during and after the infusion. Clinical observations on nausea, chills, back pain, headache, and other untoward reactions were made at frequent intervals.

Blood lipids (Fig. 1): The samples taken one-half hour after start of the protamine infusion, before the beginning of the fat emulsion infusion, did not show significant changes in optical density or free and total fatty acid content when compared with the preinfusion control. During the infusion of fat emulsion, the values for optical density, and free and total fatty acid were higher in the tests with protamine than in those without protamine. These differences were small and were statistically significant only for optical density at one hour after beginning of the fat infusion and for free fatty acid at two hours. The total fatty acid differences were not statistically significant. For the period after the fat emulsion infusion was completed, optical density was higher with protamine (statistically significant at one and two hours postinfusion), total fatty acid was higher but not statistically significantly so, and free fatty acid was higher (statistically significant at 3, 4, and 5 hours postinfusion).

The infranatant of the serum samples taken at the end of the infusion after high-speed centrifugation showed elevation of total fatty acid concentration over that of the preinfusion control sample. Free fatty acid concentration in the infranatant was the same as in whole serum, as has been previously reported by this laboratory[2] for alimentary lipemia.

Febrile response: The mean maximum elevation of rectal temperature was 1.74° F. for fat emulsion plus protamine and 1.28° F. for fat emulsion without protamine, in both instances occurring just after completion of the infusion. Mean rectal temperatures had not returned to control levels six hours after completion of the infusions. Temperature responses were calculated as fever indices by the method of Beeson.[3] Analysis of variance showed that the mean fever index with protamine, 396, was significantly higher than without prota-

mine, 311. Differences between subjects in their febrile responses were highly significant, but there was no significant difference between the mean responses to the first and second infusions.

Blood pressure, pulse, and respiration rate: During the infusion of fat emulsion without protamine, the mean value for blood pressure rose from 116/73 to 124/83 and during infusion of fat emulsion with protamine from 114/76 to 122/85. There was no significant difference in the blood pressure response with and without protamine. After cessation of fat emulsion, infusion blood pressure returned to control levels within one hour. During and following infusion of fat emulsion without protamine, mean pulse rate rose from 64 to 77 and with protamine from 65 to 81. The elevation of pulse rate persisted for six hours after the end of the infusion, and the difference between infusions with and without protamine was statistically significant. Respiration rate showed no significant change during or after infusion of fat emulsion with or without protamine.

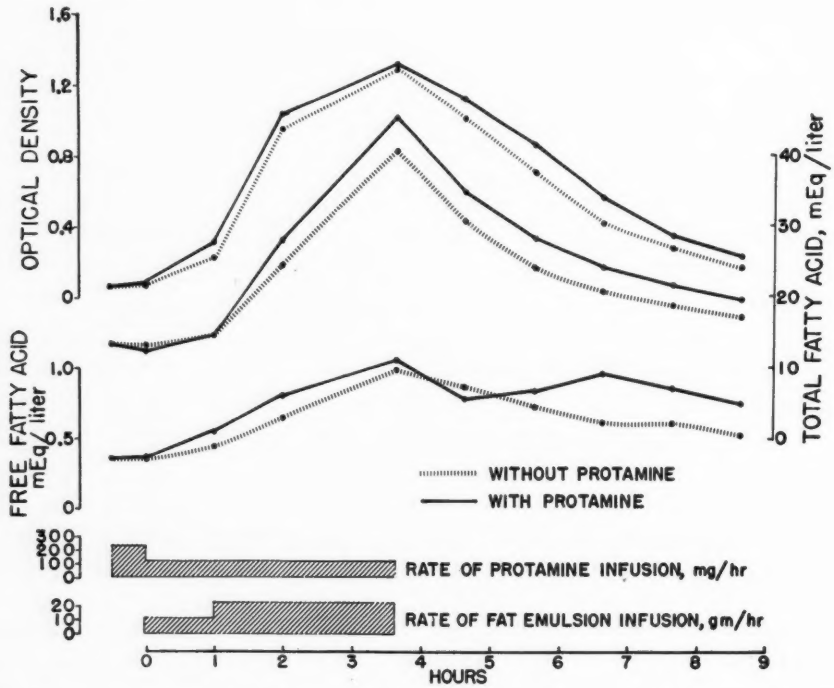

Fig. 1.—Serum lipid levels of human subjects receiving intravenous fat emulsion with and without protamine.

Side reactions: Nausea, vomiting, and backache occurred in some of the infusions with and without protamine. Eight of the 10 infusions with protamine were attended by the occurrence of urticaria. The wheals were generally surrounded by a halo and were associated with mild itching or pricking sensation. The eruption was localized mostly to the covered areas, such as the trunk and chest. There was no evidence of coryza or asthma. This reaction occurred only with the first infusion of protamine, and it subsided within 30 minutes.

A local thrombophlebitis occurred at the site of the first infusion of protamine. This was transient, lasting approximately 24 hours. It did not recur with the second infusion (i.e., with the studies on intragastric administration of fat emulsion; see below).

Back pain occurred in two subjects, one receiving the emulsion with protamine and the other the emulsion alone.

Intragastric Administration of Fat Emulsion.—The same 10 subjects used in the intravenous fat emulsion study each received two separate 500 ml. intragastric infusions of the same emulsion which was used for the intravenous study. One administration was given with protamine, one without protamine. The intragastric infusion of fat emulsion was accomplished by gravity drip through a polyethylene nasogastric tube of small diameter. The average time taken to administer the emulsion was one hour. In a few preliminary studies, more rapid administration was found to provoke vomiting in some of the subjects.

The protamine sulfate was given intravenously in the following manner: To 135 ml. of 0.9 per cent sodium chloride, 150 mg. protamine sulfate (10 mg. per milliliter) were added. Six hours after the beginning of the intragastric fat infusion, the infusion of protamine sulfate was begun. It was given at a rate of 60 drops per minute and the average time required for infusion of the 150 mg. was 30 minutes.

Blood samples were drawn according to the following schedule (times are reckoned from the beginning of the intragastric infusion of fat): (1) 5 hours; (2) 6 hours (this sample was taken immediately before starting the protamine infusion); (3) 7 hours (1 hour after beginning protamine); (4) 8 hours (2 hours after beginning protamine); and (5) 9 hours (3 hours after beginning protamine).

Blood pressure, respiration rate, pulse rate, and rectal temperature were recorded at hourly intervals.

Blood lipids (Fig. 2): Administration of protamine intravenously six hours after intragastric administration of fat emulsion produced an elevation over the control values without protamine of optical density and total fatty acid concentration during the subsequent three hours. These differences were statistically significant. Accompanying this elevation of optical density and total fatty acid concentration induced by protamine there was a *decrease* of free fatty acid concentration which was statistically significant in the first hour after protamine. When the ratio of concentration of free and total fatty acid was calculated, it was found that protamine produced a highly significant decrease in each of the three hours after its injection.

Rectal temperature: The fever indices for the days on which fat emulsion was given intragastrically were calculated. The fever indices for a day of fasting for 8 of the 10 subjects were also calculated. An analysis of variance of the data on the 8 men on whom all three values are available showed that the differences between treatments are not significant but the differences between men are. On this basis it appears to be justifiable to pool all of the data to arrive at an estimate of the mean fever index of these subjects under conditions where no treatment influencing rectal temperature was

applied. The mean value for each of the 10 men was calculated and the mean and standard deviation of these means gave values of 212.8 ± 95.5. This serves as an estimate of the fever index due to ordinary diurnal fluctation in rectal temperature and is useful in evaluating the severity of the febrile response to intravenously administered fat emulsion.

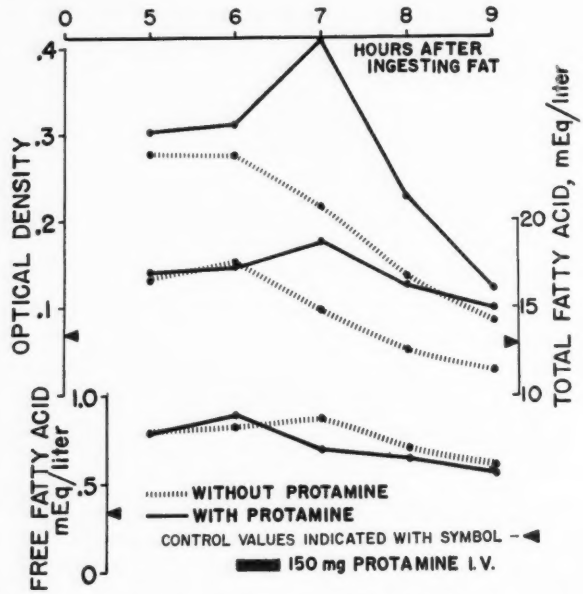

Fig. 2.—Effect of protamine on alimentary lipemia in human subjects.

STUDIES IN ANIMALS

Effect of Protamine on Blood Lipids in Rats.—Male albino rats weighing between 200 and 300 grams were fasted overnight before use. Intragastric injections were given without anesthesia with use of a No. 10 rubber ureteral catheter as a stomach tube. Intravenous injections were made into the tail veins using light ether anesthesia. Blood samples were taken under light ether anesthesia from the abdominal aorta into chilled syringes.

Effect of Protamine on Alimentary Lipemia.—

Experiment 1, Table I: Three groups of 10 rats each received 2 ml. of fat emulsion by stomach tube. Three hours later the following intravenous injections were given: Group I, 10 ml. per kilogram isotonic saline; Group II, 2 mg. per kilogram protamine; Group III, 6 mg. per kilogram protamine. Blood samples were taken one hour after the intravenous injections. Samples from pairs of rats were pooled for analysis, with 5 samples per group. Optical density, and free and total fatty acid concentration were significantly higher in the groups receiving protamine than in the group given saline. There was no significant difference in the effect of the 2 doses of protamine.

Effect of Protamine on Lipemia Resulting From Intravenous Injection of Fat Emulsion.—

Experiment 2, Table I: Two groups of 15 rats each received 1 Gm./kg. of fat intravenously in the form of 15 per cent emulsion. Fifteen minutes

TABLE I. EFFECT OF PROTAMINE ON BLOOD LIPIDS OF RATS

EXPERIMENT NO.	GROUP NO.	TREATMENT: FAT EMULSION DOSE	FAT EMULSION ROUTE	INTRAVENOUS INJECTION: SUBSTANCE	DOSE	MINUTES BEFORE OR AFTER FAT EMULSION	BLOOD LIPIDS 4 HOURS AFTER I.G. EMULSION OR 1 HOUR AFTER I.V. EMULSION (MEAN ± STANDARD DEVIATION): OPTICAL DENSITY	TOTAL FATTY ACID	FREE FATTY ACID
1.	I	2 ml.	I.G.	Saline	10 ml./kg.	+180	0.166 ±0.022	9.36 ±0.88	0.36 ±0.24
	II	2 ml.	I.G.	Protamine	2 mg./kg.	+180	0.474* ±0.224	21.32† ±3.99	0.74† ±0.10
	III	2 ml.	I.G.	Protamine	6 mg./kg.	+180	0.591† ±0.173	22.42† ±2.80	0.68* ±0.08
2.	I	1 Gm./kg.	I.V.	Saline	10 ml./kg.	−15	1.260 ±0.136	32.18 ±11.14	
	II	1 Gm./kg.	I.V.	Protamine	2 mg./kg.	−15	1.379* ±0.091	37.35 ±8.96	
3.	I	1 Gm./kg.	I.V.	Saline	10 ml./kg.	0	1.096 ±0.195	28.62 ±9.06	1.094 ±0.371
	II	1 Gm./kg.	I.V.	Protamine	6 mg./kg.	0	0.208† ±0.043	17.50* ±1.68	0.656* ±0.053
4.	I	1 Gm./kg.	I.V.	Saline	10 ml./kg.	0	1.060 ±0.211	24.1 ±6.84	1.25 ±0.31
	II	1 Gm./kg.	I.V.	Protamine	6 mg./kg.	−15	1.400† ±0.058	47.2† ±4.31	1.29 ±0.16
	III	1 Gm./kg.	I.V.	Protamine	6 mg./kg.	0	0.146† ±0.036	13.5† ±2.51	0.69† ±0.08

I.G. = Intragastric.
I.V. = Intravenous.
*Statistically significantly different from saline control ($P<0.05$).
†Statistically highly significantly different from saline control ($P<0.01$).

before injection of emulsion, Group I received 10 ml./kg. of saline intravenously and Group II received 2 mg./kg. of protamine intravenously. Blood samples were taken one hour after injection of fat emulsion. The optical density and total fatty acid concentration were slightly higher in the group which received protamine (statistically significant for optical density but not for total fatty acid).

Experiment 3, Table I: Two groups of 10 rats each received 1 Gm./kg. of fat emulsion intravenously. Immediately before injection of the fat emulsion (through the same needle used to inject the fat emulsion), Group I received 10 ml./kg. of saline and Group II 6 mg./kg. of protamine. Blood samples (pooled for pairs of rats) were taken one hour after injection of fat emulsion. The group treated with protamine showed significantly *lower* optical density, and free and total fatty acid concentrations than the saline group.

Experiment 4, Table I: Three groups of 10 rats each received 1 Gm./kg. of fat as emulsion intravenously. Immediately before injection of the fat emulsion (through the same needle), Group I received 10 ml./kg. of saline and Group III 6 mg./kg. of protamine. Group II received 6 mg./kg. of protamine intravenously (separate injection) 15 minutes before the fat emulsion. The group given protamine immediately before fat emulsion had much lower optical densities, and free and total fatty acid concentrations than the saline group (statistically highly significant). The group given protamine 15 minutes before the injection of emulsion had much higher optical densities and total fatty acid concentrations (statistically highly significant), but the free fatty acid concentration was not significantly different from the saline control group.

DISCUSSION

From the results of the present study it would appear that the effectiveness of protamine in elevating blood lipid levels is greater with alimentary lipemia than with lipemia induced by intravenous injection of fat emulsion. Furthermore, in the studies on rats, it was found that injection of protamine immediately before fat emulsion speeded rather than delayed the removal of the fat from the blood.

The elevation of blood lipid produced by protamine is accompanied by a change in free fatty acid concentration which is smaller than would be expected if the elevation had been caused by alimentary lipemia. This is particularly striking in the case of protamine and alimentary lipemia in the human subjects where a fall in free fatty acid concentration accompanied the rise in total fatty acid concentration produced by protamine. If one postulates (a) that the removal of fat from the blood under normal circumstances involves the action of a substance comparable to heparin-induced clearing, and (b) that the mechanism of action of clearing factor is lipolytic,[5] then one can hypothesize that protamine acts as an inhibitor of clearing factor, reducing the rate of release of free fatty acid. By the partial blocking of this step in chylomicron degradation, it can be hypothesized that protamine produces a delay in removal of fat from the blood.

The acceleration of fat removal from the blood when protamine and fat emulsion were injected simultaneously in rats is probably due to agglutination

of fat particles by the high concentration of protamine.[3] This explanation is supported by the histologic findings in animals so treated. Animals receiving protamine and fat emulsion as separate injections 15 minutes apart show accumulations of finely dispersed fat in the lung, liver, and spleen, whereas animals which have received simultaneous injections of the two substances show large globules of fat in these organs.

A detailed presentation of the findings presented in this paper is given in Medical Nutrition Laboratory Report No. 164, April 22, 1955.

SUMMARY AND CONCLUSIONS

In human subjects: (a) Simultaneous intravenous administration of protamine and fat emulsion slightly delayed the rate of removal of the fat from the blood and slightly increased the febrile response attending the infusion of the fat, and (b) intravenous infusion of protamine during alimentary lipemia produced a moderate elevation of blood lipid as compared with the control values without protamine.

In rats: (a) Protamine given intravenously increased the lipemia resulting from feeding fat; (b) when the injection of protamine immediately preceded injection of fat emulsion, removal of fat from the blood was markedly increased; but when injection of protamine preceded by 15 minutes injection of fat emulsion, marked delay in removal of fat from the blood occurred.

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THE EFFECT OF PROGESTERONE AND ANHYDROHYDROXY-PROGESTERONE ON NITROGEN BALANCE

LAURENCE H. KYLE, M.D., AND WALTER C. HESS, PH.D.
WASHINGTON, D. C.

NUMEROUS studies have established the importance of progesterone in the development of the female reproductive tract and growth of sex-linked tissues, but this hormone has been considered relatively inert with respect to systemic effects. In 1942 Albright[1] noted that the administration of progesterone to a patient with Cushing's syndrome caused little change, except for slight retention of nitrogen, but in a later communication he expressed the opinion that progesterone might act unfavorably on nitrogen balance.[2] In 1944 Abels and Dobriner[3] demonstrated a significant degree of negative nitrogen balance in a single subject during the daily administration of 100 mg. of progesterone. A similar response in patients with rheumatoid arthritis treated with either progesterone or anhydrohydroxyprogesterone has been reported from this laboratory.[4] Recently Landau and associates[5] reported the occurrence of significant nitrogen loss during treatment with progesterone in normal subjects and a more marked catabolic effect in patients with adrenocortical deficiency. The present study concerns expansion of our previous observations in an attempt to define both the regularity and mechanism of negative nitrogen balance during treatment with progesterone and anhydrohydroxyprogesterone.

MATERIALS AND METHODS

The subjects consisted of male and female patients with rheumatoid arthritis, ranging in age from 14 to 53 years, and an 8-year-old girl with congenital adrenal hyperplasia. They were hospitalized on the metabolic ward and given a diet constant in nitrogen and calories. Diets were calculated on the basis of 10 Gm. of nitrogen and 1,600 calories per 1.73 square meter of body surface for basal requirements, with additions based on the amount of activity during the study period. Urine specimens collected for each 24-hour period were analyzed for nitrogen by a micro-Kjeldahl technique. Stool nitrogen was assumed to be 1.3 Gm. per day.[6] Urinary 17-ketosteroids were measured daily or at frequent intervals throughout the study by the method of Holtorff and Koch.[7] Total circulating eosinophiles were counted by a modification of the Randolph technique.[8] Serum cholesterol partition was conducted by the method of Hess.[9] Standard methods were used for differential white blood cell counts and for measurement of blood sugar. After a control period of at least eight days, progesterone was given intramuscularly in divided doses; anhydrohydroxyprogesterone was administered orally every eight hours.

RESULTS

Table I summarizes the changes in nitrogen excretion during 7 courses of progesterone and 3 courses of anhydrohydroxyprogesterone in 8 patients with

From the Departments of Medicine and Biological Chemistry, Georgetown University School of Medicine and Georgetown University Hospital.

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rheumatoid arthritis. The average daily urinary nitrogen excretion during therapy is compared with the excretion during a 6- to 8-day control period, started after the subject had attained nitrogen equilibrium. In all but one of 10 studies, nitrogen excretion rose during therapy, but in 3 instances the catabolic effect was minimal. No significant difference was noted between male and female patients.

TABLE I. CHANGE IN NITROGEN EXCRETION

CASE	SEX	DRUG	DOSE (MG.)	NITROGEN INTAKE (GM./DAY)	URINARY NITROGEN EXCRETION		
					BEFORE	DURING	CHANGE
1. (K. G.)	M	Progesterone	50	11.6	12.0	15.9	−3.9
		Anhydrohydroxyprog.	300	10.6	10.0	14.5	−4.5
2. (H. C.)	M	Progesterone	50	11.2	8.1	11.7	−3.6
3. (C. T.)	M	Progesterone	100	9.0	7.4	8.7	−1.3
4. (L. G.)	M	Progesterone	100	12.5	12.4	13.2	−0.8
		Anhydrohydroxyprog.	300	12.5	12.4	12.0	+0.4
5. (G. J.)	F	Progesterone	50	7.2	6.7	7.2	−0.5
6. (M. P.)	F	Progesterone	100	13.9	12.4	13.2	−0.8
7. (V. S.)	F	Progesterone	100	11.9	9.0	11.1	−2.1
8. (M. S.)	F	Anhydrohydroxyprog.	300	14.5	16.5	19.1	−2.6

TABLE II. CHANGE IN URINARY 17-KETOSTEROID EXCRETION

CASE	SEX	DRUG	DOSE (MG.)	URINARY 17-K.S. (MG./DAY)		
				BEFORE	DURING	AFTER
1. (K. G.)	M	Progesterone	50	5.6	5.9	5.5
		Anhydrohydroxyprog.	300	5.5	6.4	—
2. (H. C.)	M	Progesterone	50	6.7	5.2	5.4
3. (C. T.)	M	Progesterone	100	5.5	5.0	—
4. (L. G.)	M	Progesterone	100	6.1	7.2	7.5
5. (G. J.)	F	Progesterone	50	4.5	6.1	—
6. (M. P.)	F	Progesterone	100	7.6	7.9	—
		Anhydrohydroxyprog.	300	7.6	8.2	—
7. (V. S.)	F	Progesterone	100	6.0	6.9	—
		Progesterone	200	6.1	6.5	—
8. (M. S.)	F	Anhydrohydroxyprog.	300	6.3	7.1	—

Excretion of urinary 17-ketosteroids was measured during 8 courses of progesterone and 3 courses of anhydrohydroxyprogesterone in the 8 patients. Table II expresses the average value of several determinations during the control period and the average level of excretion during treatment. In no case was a significant change observed. However, the low levels of 17-ketosteroid excretion noted in these chronically ill patients would tend to dampen slight changes. In 3 patients measurements were continued for fourteen days after cessation of therapy. There was no evidence that progesterone exerts any delayed effect on urinary 17-ketosteroid excretion.

Fig. 1 illustrates nitrogen balance, 17-ketosteroid excretion, and total eosinophil counts in a 53-year-old man with rheumatoid arthritis. Intramuscular ACTH caused a marked nitrogen loss, significant elevation of urinary 17-ketosteroids, and eosinopenia. Progesterone was started immediately following the ACTH, and this treatment was associated with significant negative nitrogen balance of lesser magnitude than that induced by ACTH. Neither eosinophils nor urinary 17-ketosteroids were affected by the administration of progesterone.

Fig. 2 depicts the metabolic effects of a number of hormonal preparations in a 40-year-old man with rheumatoid spondylitis. Pregnenolone, 200 mg. per day, caused no appreciable change in nitrogen balance, but there was a slight increase in nitrogen excretion when the dose was increased to 300 mg. per

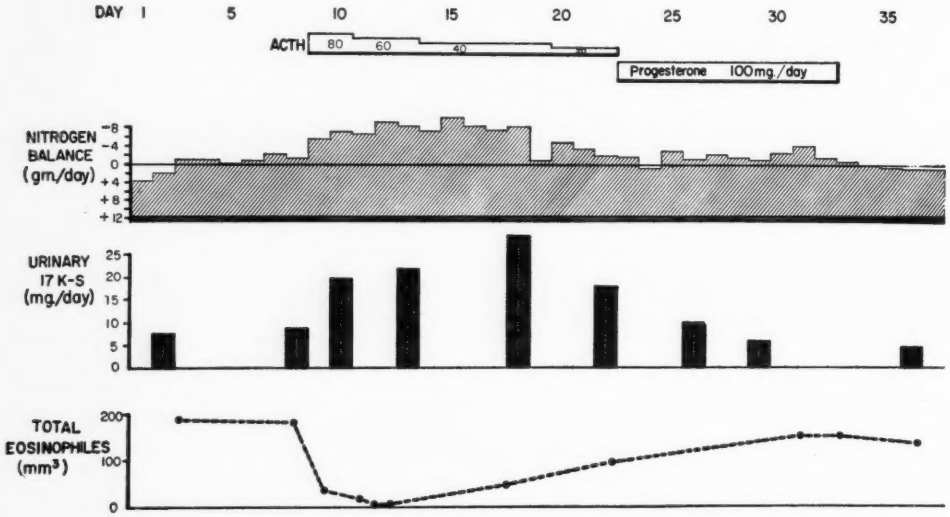
DAY 1 5 10 15 20 25 30 35
ACTH 80 60 40
Progesterone 100mg./day
NITROGEN BALANCE (gm./day)
URINARY 17 K-S (mg./day)
TOTAL EOSINOPHILES (mm³)

Fig. 1.—Case 9. Rheumatoid arthritis. Effect of ACTH and progesterone.

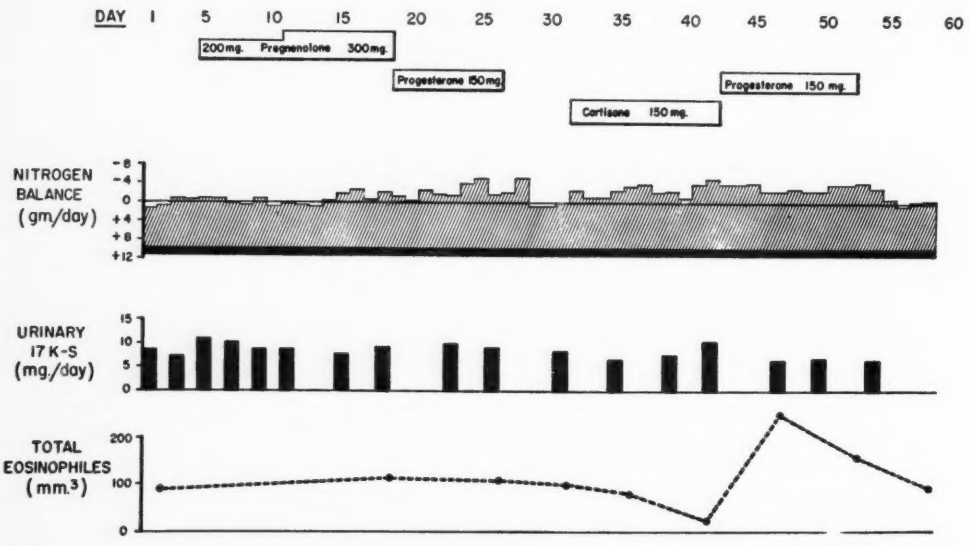
DAY 1 5 10 15 20 25 30 35 40 45 50 55 60
200mg. Pregnenolone 300mg.
Progesterone 150mg.
Progesterone 150 mg.
Cortisone 150 mg.
NITROGEN BALANCE (gm/day)
URINARY 17 K-S (mg./day)
TOTAL EOSINOPHILES (mm.³)

Fig. 2.—Case 10. Rheumatoid arthritis. Comparison of progesterone with other steroid hormones.

day. Definite negative nitrogen balance resulted from the administration of 150 mg. of progesterone. The same dose of intramuscular cortisone, given after a short rest period, caused a slightly greater unfavorable effect on nitrogen balance. When progesterone was given again immediately following cessation

of cortisone therapy, nitrogen excretion exceeded that which was observed during the first course of progesterone. Although persistent cortisone effect might account for nitrogen loss during the initial portion of the second course of progesterone, the rapid rise in eosinophils indicates that such an effect, if present, was of brief duration. No significant change in 17-ketosteroid excretion occurred during the entire period of study, and the number of circulating eosinophils varied from the control values only during and immediately following the use of cortisone.

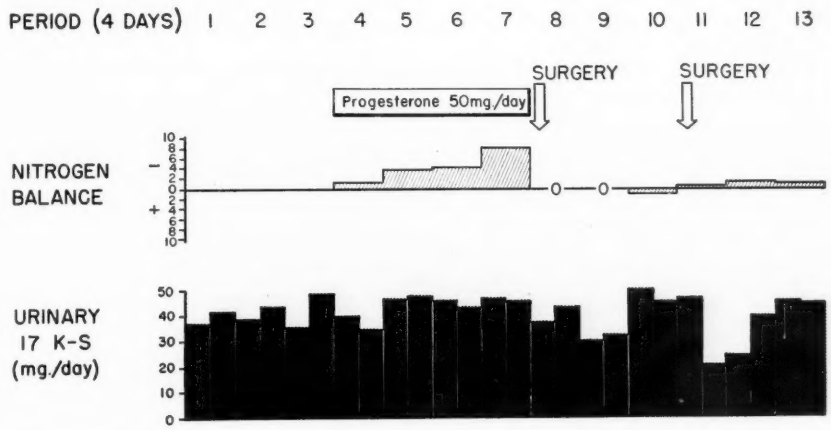

Fig. 3.—Case 11. The effect of progesterone in congenital adrenal hyperplasia.

Fig. 3 illustrates the response to progesterone in an 8-year-old girl with congenital adrenal hyperplasia. Here also may be noted a definite catabolic effect, but the markedly elevated urinary 17-ketosteroids were not affected by progesterone. The decrease in urinary 17-ketosteroids which followed discontinuation of progesterone therapy was most probably the result of the laparotomy performed at this time, for a similar decrease in excretion occurred after a second operation (amputation of clitoris), two weeks after cessation of progesterone therapy.

None of the other determinations, such as blood cholesterol partition, differential white cell counts, and FBS or glucose tolerance tests, showed changes indicative of a corticoid influence.

DISCUSSION

These studies lend support to previous observations that progesterone exerts a small but definitely unfavorable effect on nitrogen balance. This influence is seen in both sexes and is equally as great in the older subject as in the one in the active reproductive years. While several mechanisms could be advanced to explain this catabolic effect, only 4 appear to warrant consideration: (1) stimulation of production of glyconeogenic corticoids, (2) conversion of progesterone to a steroid with antianabolic properties, (3) inhibition of production of the adrenal anabolic hormone (''N'' hormone), and (4) a direct effect at the tissue level.

This study provides no evidence that progesterone exerts a stimulant effect on the adrenal gland, and, in view of the observations of Landau and associates that the protein catabolic effect of progesterone occurs in patients with Addison's disease, this possible mode of action can be dismissed. It seems equally improbable that progesterone is converted to a corticoid with antianabolic properties, for these studies revealed no changes in such indicators as eosinophils, differential white cell counts, blood sugar, glucose tolerance tests, or cholesterol partition suggestive of increased corticoid production.

The possibility that progesterone might inhibit the production of adrenal "N" hormone, thus provoking an imbalance between anabolic and antianabolic influences in the body, bears some consideration. A certain amount of circumstantial evidence, summarized by Reifenstein, Forbes, and Donaldson,[10] suggests that luteinizing hormone (L. H.) stimulates the production of adrenal androgen. Evidence contrary to this hypothesis, presented by Sayers and Sayers,[11] has been strengthened by the observation that ACTH appears to be responsible for the elaboration of all adrenal hormones excreted as 17-ketosteroids. If, however, luteinizing hormone were responsible, at least in part, for secretion of adrenal androgen, it is logical to presume that progesterone, by inhibition of L. H. production, could induce a catabolic effect. However, the fact that progesterone induces a negative nitrogen balance in patients with Addison's disease and the response observed when progesterone was given immediately after cortisone, when there should exist a state of temporary adrenal hypofunction, render this concept extremely unlikely. The fact that urinary 17-ketosteroid excretion was not affected by progesterone serves as additional evidence that the protein catabolic effect is not due to suppression of adrenal androgen production.

Consequently it seems most probable that progesterone exerts its effect at the tissue level. This could be a direct catabolic effect on protein tissue or the result of inhibition of action of endogenous anabolic hormones. While animal studies by Pincus and Dorfman[12] demonstrated that progesterone acts as a potent inhibitor of the stimulant effect of testosterone on seminal vesicles, ventral prostate, and levator ani growth, the studies reported here cannot separate the two possible sites of action.

SUMMARY

The administration of progesterone and anhydrohydroxyprogesterone induced nitrogen loss in 13 of 14 courses of treatment, which was of significant degree in 10 of the studies. This catabolic effect was noted when progesterone was given immediately following the administration of either ACTH or cortisone. There was no significant change in urinary 17-ketosteroids nor in any of the indicators of an increase in adrenal cortical activity. In one patient with congenital adrenal hyperplasia, progesterone induced loss of nitrogen but did not depress the excessive excretion of 17-ketosteroids. The unfavorable effect of progesterone on nitrogen balance appears to be mediated at the tissue level.

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OBSERVATIONS ON THE EFFECTS OF INTRA-ARTICULAR PHENYLBUTAZONE

DAVID H. NEUSTADT, M.D., LOUISVILLE, KY., AND OTTO STEINBROCKER, M.D., NEW YORK, N. Y.

IN PREVIOUS therapeutic observations on phenylbutazone (Butazolidin) administered systemically in various rheumatic conditions, its outstanding effect was found to be the relief of pain.[1] Specific anti-inflammatory action was suggested but not definitely demonstrated. This study was undertaken to observe: (1) the clinical effects of phenylbutazone when injected intra-articularly and, at the same time, (2) to investigate the possibility that antirheumatoid or anti-inflammatory properties of phenylbutazone might be demonstrated when it is administered at the local tissue level. The clinical responses and synovial fluid changes following the intra-articular injection of this compound therefore constitute the basis of this report.

METHODS AND MATERIALS

Phenylbutazone was given intra-articularly in a series of 33 patients. Eighteen patients suffered from rheumatoid arthritis, 11 from osteoarthritis, and 4 presented various allied rheumatic conditions. A 20 per cent solution of 1 Gm. of phenylbutazone (with Xylocaine as a constituent), for intramuscular use, was employed. The size of the injections and the intervals between them were determined by the patients' responses. The average dosage varied from 3 to 5 ml. of the solution. The minimum interval between injections was one week. The usual period was two weeks but ranged from one week to six weeks in those patients who received repeated injections. All patients were given intra-articular infiltrations of physiologic saline and/or 1 per cent procaine solution for control observations, prior to receiving phenylbutazone. The technique of administration of the drug was regulated according to the needs of the involved joint. Those articulations containing fluid were aspirated prior to the injection of the material. In those patients in whom serial synovial fluid observations were conducted, the fluid was examined at weekly intervals, with the exception of a small number of patients in whom fluid was removed two and one-half days (60 hours) after injection. Response was assessed according to subjective and objective criteria (Table I, footnote).

Methods for appraisal of objective joint improvement included circumferential measurement of the joint, recording of changes in ranges of motion, and observations of surface temperature and/or color change when present.

RESULTS

The clinical results are presented in Table I. A total of 45 injections was given to 18 patients with rheumatoid arthritis. Seven members of this group derived major improvement, 7 minor improvement, and 4 failed to benefit.

From the Rheumatology Departments, Lenox Hill Hospital and the Hospital for Joint Diseases, New York, N. Y.

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Eleven patients with osteoarthritis received 34 intra-articular injections. One was markedly benefited, 7 slightly to moderately benefited, and 3 failed to respond. The other musculoskeletal disorders in the series were not significantly influenced. These included an olecranon bursitis in a rheumatoid arthritic, a swollen knee in a patient with ankylosing spondylitis with peripheral joint involvement, a tendovaginitis of the dorsum of a wrist, and an acutely swollen knee in a case of Reiter's syndrome. Each of these received only one injection, except the patient with the Reiter's disorder who received two injections. The patients with the olecranon bursitis and ankylosing spondylitis are included in the rheumatoid group in Table I. An acute synovitis of the knee resolved rapidly and completely after one injection, for a follow-up period of four months, an observation of dubious significance in only one case.

TABLE I. RESULTS FROM INTRA-ARTICULAR PHENYLBUTAZONE

DIAGNOSIS	TOTAL NO. OF CASES	TOTAL NO. OF INJECT.	SITES WITH NO. OF INJECT. TO EACH	AMOUNT OF AGENT	COMPLETELY* RESOLVED	GREATLY† IMPROVED	APPRECIABLY‡ (SL.-MOD.)	FAILURES§	DURATION OF BENEFICIAL EFFECT (EXTREMES)
Rheumatoid arthritis	18	45	Knees 35 Shoulder 2 Wrist 4 Olecr. 1 Hip 2	3-5 ml. (knee, hip) 1¼-2½ ml. (Others)	2	5	7	4	2 wks. 11 mo.
Osteoarthritis	11	34	Knees 22 Shoulder 1 Hip 5	2-5 ml.	1	0	7	3	2 wk. 3 mo.
Totals	29	79							

*Completely resolved: Relief of pain, resolution of swelling, and return of normal function for a minimum period of 6 months.

†Greatly improved: Marked relief of symptoms, decreased swelling, with improvement in function for a minimum period of 1 week or more.

‡Appreciably improved: Pain relief without objective changes for a minimum period of 1 week.

§Failures: Any lesser response than the above, same or worse.

The most favorable results were noted in the more acutely active and painful joints with increased fluid. The poorest effects were observed in the chronic, mildly painful joints with presumably low-grade inflammatory, clinical activity and thickened, boggy synovia. Of fourteen rheumatoid knees treated, seven showed significant objective evidence of improvement.

Study of the synovial fluid was confined to the cytologic examination. Total, red, and differential white cell counts were done before and after all injections in 10 patients. These findings are shown in Table II. Five patients in the small number tested appeared to have a significant decrease in the white cell count of the synovial fluid one to two weeks following the introduction of phenylbutazone into the joint. When synovial fluid was removed shortly after the joint had been injected, the morphology of the cells was so altered that a differential count was impossible. The addition of phenylbutazone to synovial fluid in vitro caused the same disintegration of the cells.

TABLE II. SYNOVIAL FLUID CYTOLOGY STUDIES

NAMES	DIAGNOSIS	NO. OF INJECTIONS	JOINT FLUID CYTOLOGY								
			BEFORE INJECTION			AFTER FIRST INJECTION			AFTER TOTAL NO. INJECTIONS		
			W.B.C.	POLYS.	LYMPHOS. MONOS.	W.B.C.	POLYS.	LYMPHOS. MONOS.	W.B.C.	POLYS.	LYMPHOS. MONOS.
C. H.	Osteo.	Knee-1	500	40%	58	0					
B. T.	Osteo.	Knee-3	63	100%	0	270	85	14			
L. B.	Osteo.	Knee-5	15	90%	10	162	58	42			
E. B.	Osteo.	Knee-2	108	12%	88	68	14	86			
C. J.	Osteo.	Knee-6	730	90	10	420	CD*	CD*	10	CD*	CD*
A. V.	Rheumatoid	Knee-1	84,400	86	14	37,600	CD*	CD*			
J. B.	Rheumatoid	Knee-4	1,600	82	16	1,500	74	20	5,000	63	27
A. T.	Rheumatoid	Knee-3	56,000	88	12	24,000	76	24	2,700	90	10
A. M.	Rheumatoid	Knee-4	35,443	51	48	29,900	49	42	19,300	66	31
O. K.	Rheumatoid	Knee-1	4,300	33	56	2,600	48	43			

*CD=Cells Disintegrated.

UNTOWARD EFFECTS

No systemic effects of administering phenylbutazone by the intra-articular route were apparent clinically. A subjective sensation, usually described by the patient as "burning," occurred in approximately one-half of the patients during the introduction of the material, occasionally of great but transient severity. In the relatively "dry" joint, as contrasted with those containing fluid, the local discomfort was more marked. It subsided within a few minutes to several hours in all instances. In one rheumatoid patient, when 3 ml. of the solution was injected into the knee without the preliminary use of smaller quantities, an acute effusion developed requiring aspiration twenty-four hours later. No untoward effects occurred thereafter with smaller doses.

DISCUSSION

Although the therapogenic basis for the effects of phenylbutazone remains unknown, it has been suggested by certain experimental laboratory studies that there may be a definite anti-inflammatory action.[2, 3] Payne and co-workers,[4] using the serum protein polysaccharide ratio (PR) as an index of clinical activity in rheumatoid arthritis, have reported that phenylbutazone produces a fall in this level. Histologic studies of the synovial membrane of a knee before and after treatment with oral phenylbutazone in a patient with rheumatoid arthritis revealed evidence of a marked decrease in the signs of inflammation.[5] A report of the effects of intra-articular phenylbutazone in osteoarthritis of the hips described relief of pain and relaxation of muscle spasm.[6] However, the possibility of a systemic effect, if the drug failed to enter the hip joint, must be considered. In a recent study which just has come to our attention, phenylbutazone was given intra-articularly to 11 patients with rheumatoid arthritis.[7] A single injection of 1 to 3 ml. of the agent was instilled into a joint and the effect compared with saline, procaine, and hydrocortisone. Tabulation of the clinical results revealed improvement in 9 patients, as compared with control instillations of saline and procaine. The effects were poor and of short duration compared to those of compound F. Repeated injections were not administered. Criteria for objective improvement were not defined.

In our small series of patients with rheumatoid and osteo-arthritis, no constant anti-inflammatory effect was demonstrated. It is relevant, perhaps, that the therapeutic response to intra-articular phenylbutazone in this admittedly small group closely approximates our results in a larger series of patients receiving hydrocortisone by the same route.[8]

The frequency of benefit from the intra-articular injection of phenylbutazone suggests some definite, if not generally predictable, local therapeutic action. This effect may arise from an anti-inflammatory property of the compound, more noticeable at the tissue level than we observed by systemic administration of the drug. It may happen, nevertheless, that some as yet undefined physicochemical reaction of therapeutic proportions may follow the introduction of a variety of substances into an inflamed joint. The possibility that the beneficial response to phenylbutazone originates from such a nonspecific mechanism cannot be excluded by this clinical study.

SUMMARY AND CONCLUSIONS

1. An experimental study has been conducted to observe the effect of phenylbutazone when given directly into the joints.

2. Eighty-seven injections of phenylbutazone were administered intra-articularly in a series of 33 patients with various musculoarticular disorders, including 18 with rheumatoid arthritis and 11 with osteoarthritis.

3. Observations are presented on the changes in the synovial fluid cytology following the intra-articular introduction of phenylbutazone.

4. Of 18 patients with rheumatoid arthritis, given a total of 45 intra-articular infiltrations of the drug, 7 derived major improvement, 7 minor, and 4 did not benefit. Of 11 patients with osteoarthritis receiving 34 intra-articular injections, 1 showed major improvement, 7 slight to moderate improvement, and 3 failed to respond. Of 4 patients with related conditions, none was benefited.

5. The respectable number of beneficial responses strongly suggest some anti-inflammatory effect, but the possibility of a nonspecific physicochemical therapeutic mechanism cannot be excluded from these limited observations.

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STUDY OF RECORDED NOISE IN NORMAL AND PATHOLOGIC KNEE JOINTS OF HUMAN SUBJECTS

JOHN HUNTER, PH.D., AND H. C. BROOKS, M.D.
TORONTO, ONTARIO

WITH THE TECHNICAL ASSISTANCE OF A. J. BROCK

AUSCULTATION in joint diagnosis has been attempted by many investigators, beginning with Hueter[3] in 1885. Satisfactory diagnostic results with auscultation of the various parts of the vertebral column and particularly the sacroiliac joint were reported by Ludloff[4] in 1906. Erb,[2] in 1933, and Ekensten,[1] in 1952, have reported the successful registration of joint noises using electronic means. Technical improvements make the process of detecting, amplifying, and recording joint noise relatively simple. The present work was undertaken to determine whether the information that the method will provide would justify introduction of the technique as a diagnostic aid.

METHOD

The apparatus used for recording joint sounds is illustrated diagrammatically in Fig. 1.

Articular noises were detected with a crystal type microphone* 1¼″ in diameter. The frequency response of this microphone is 200 c.p.s. flat to 2,000 c.p.s. The microphone, cushioned by a ½″ sponge rubber pad and backed with a fiber plate, was attached by elastic straps to the knee joint in the medial or the lateral position. The microphone output was amplified by a push-pull 4 stage direct coupled amplifier,† with a gain of 3,500. The frequency response of the amplifier is 2,000 c.p.s. flat. The output of the amplifier was tapped to a double beam oscillograph.‡ The oscilloscope beams, after further amplification (×2), were photographed at film speed of 1.5 cm. per second.§ The total amplification of the recorded joint noise was routinely 7,000.

Essentially, the same information may be obtained by matching the microphone impedance to that of a standard ECG oscillograph. The usual fixed camera speed for an electrocardiogram is 25 mm. per second. Types of ECG machines with limited frequency response would not be adequate. To adapt existing ECG models for this type of recording would be relatively simple.

The test subjects were supported by a frame 7′ × 4′ × 4′ built of 1″ o.d. pipe (Fig. 2). To the top of the frame were bolted two 1″ angle irons 6″ apart. These supported shafts with a pulley 1½′ from the front of the frame directly over the subject, and a pulley protruding 3″ beyond the back of the frame. Bowden cable (¼″), stretched over the pulleys, held 2 rings which supported the subjects from the axillae. A seat was also

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*Brush Electronic Co., Cleveland, Ohio.
†Edin Company, Inc., Worcester 8, Mass.
‡A. C. Cossor Ltd., London, Eng.
§Cossor Model 1428 oscillograph camera.

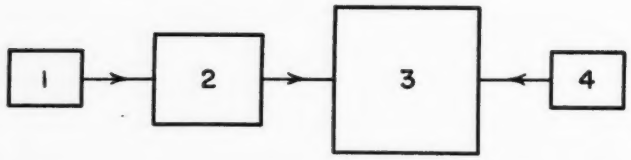


Fig. 1.—Block diagram of apparatus used for recording joint sounds and position: *1*, Microphone (applied to knee joint); microphone used was a crystal type microphone 1¼" in diameter, ¼" thick. *2*, Edin direct coupled amplifier Model #8105. *3*, Cossor double beam oscillograph Model 1049 d.c. oscillograph. Maximum gain 900 with a frequency response from 0-100 KCS ± 15%. Any d.c. oscilloscope with these specifications could be used. *4*, Disc commutator.

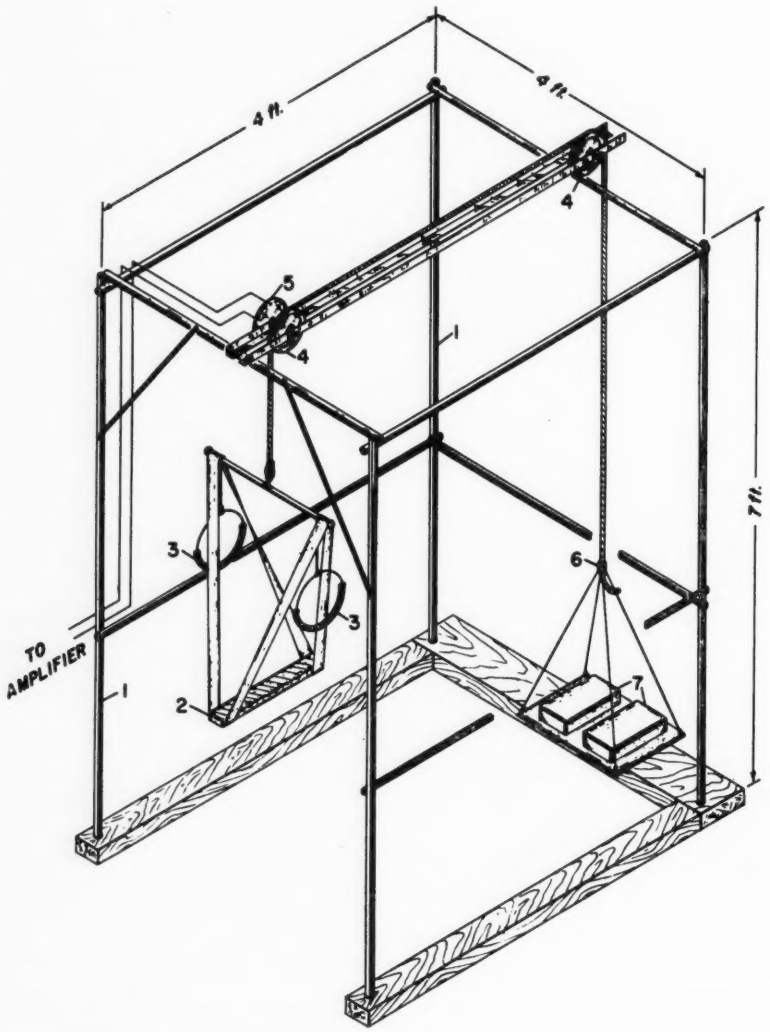


Fig. 2.—System for recording sounds and flexion of knee joints using counterweights. *1*, Supporting frame; *2*, seat; *3*, padded rings; *4*, pulleys; *5*, disc commutator; *6*, cable adjustment; *7*, counterweights.

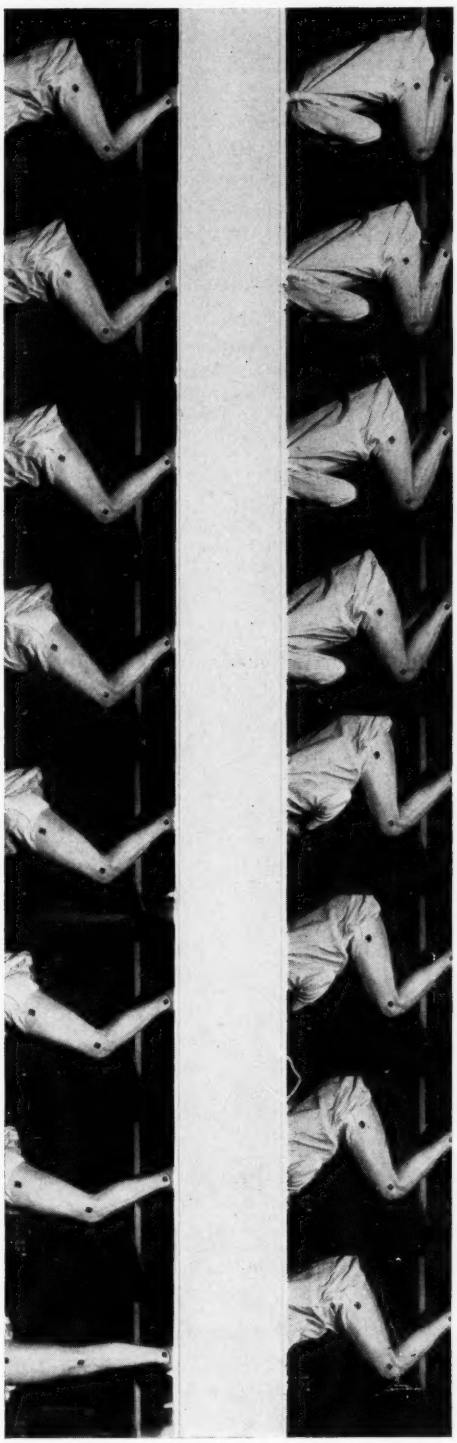


Fig. 3.—Angles of flexion of knee joint in sequence: 1st row: 173° 140° 122° 111° 99° 90° 87° 80°; 2nd row: 80° 76° 73° 68° 58° 46° 32° 25°.

suspended from the cable to help maintain support. On the other end of the cable, a wooden platform (18″ × 18″) was attached with an adjustable cable tightener to hold sand bags counterweighting the subject.

The angles of flexion of the knee were recorded by deflections of a second pen on the Pen Exciter and the second beam of the oscillograph. A Bakelite disc, 6″ in diameter, with 8 pairs of brass contacts 45 degrees apart was mounted beside the front pulley over the subject. A brass spring contact from the pulley completed a 6 volt d.c. circuit as it rotated.

To measure the angles of flexion in degrees, pictures in sequence were taken with a Robot camera at all contact points (Fig. 3). The angles were measured with a goniometer, from the film projected through a Kodagraph film reader.

The test subjects were supported in the frame and electrically grounded. Each subject carried out a knee bend and returned to the normal standing position, counterbalanced with half his body weight and also with a 10 pound counterweight. These operations were repeated with the microphone on the medial and the lateral aspect of the right and of the left knee. An effort was made to keep the rate of bending uniform for each individual. Any particular noises produced by flexion of the knee joint were recorded.

RESULTS

In this study the records of 27 subjects are divided into 5 groups.

1. Normal (a) adult	13 subjects
(b) children	3 subjects
2. Osteoarthritis	3 subjects
3. Rheumatoid arthritis	6 subjects
4. Subacute rheumatic arthritis (in remission)	1 subject
5. Traumatic arthritis (recovered clinically)	1 subject

The clinical diagnoses of rheumatoid arthritis and osteoarthritis were established prior to these investigations by the medical staff of Sunnybrook Hospital, Department of Veterans' Affairs, Toronto.

1. *Normal.*—

a. Adult (Fig. 4, *A*): Of the 12 "normal" adult records, 11 showed little noise above background, with occasional single spikes corresponding to the familiar tendon and joint snaps. Two subjects produced some noise, although they had no immediate complaint concerning the knees.

In one of these, inquiry revealed that he had been pensioned for ten years for osteoarthritis of the lumbar spine. The other subject, whose record showed abnormal tracings in one knee had a history of trauma to that knee in a minor aircraft mishap in 1944. The knee, though usually symptom-free, has responded to slight trauma and exposure to cold by swelling and tenderness.

b. Children (Fig. 4, *B*): Two of these produced records with practically no noise above background. The other child showed some noise production. For this child, no complaints nor relevant history could be discovered.

To summarize the records from 12 "normal" individuals, thirteen gave practically noiseless records. Two adults had noisy records and their history

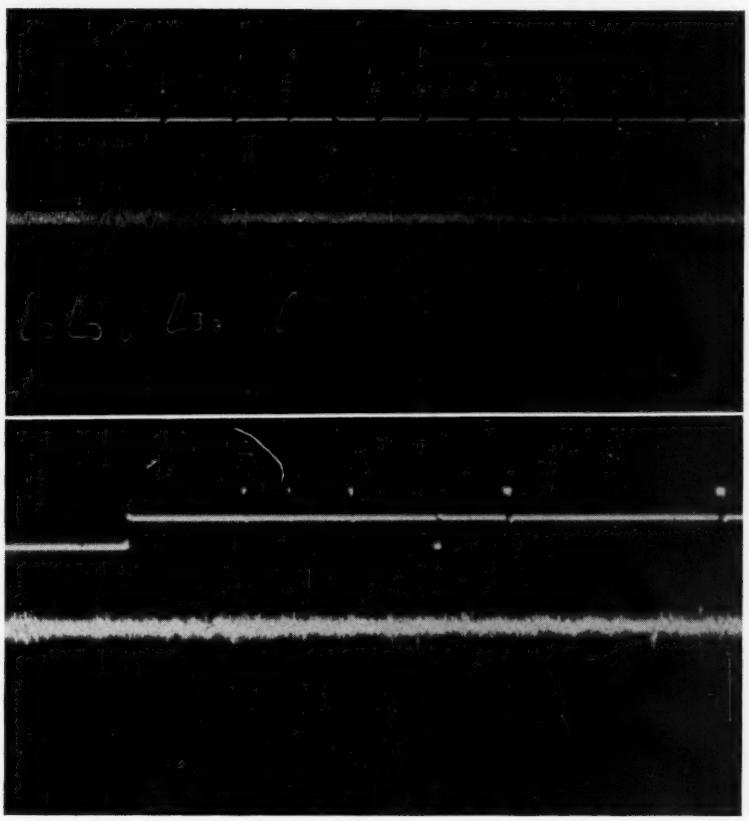
indicated probable abnormality. The remaining child produced a noisy record for no apparent reason. It is possible that this child's future clinical history will offer some explanation.

2. *Osteoarthritis* (Fig. 5, *C*).—There were 3 patients with this diagnosis.

Patient 1: This patient produced records indistinguishable from the normal, though he complained a great deal about everything he was asked to do.

Patient 2: This subject had generally stiff painful joints, making movement quite slow. He produced noise in his right knee of amplitude about five times normal and over a range of twenty degrees of flexion. It was this knee which was subjectively more troublesome.

A.



B.

Fig. 4.—Normal records: *A*, Adult, *B*, Child.

Patient 3 (Fig. 5, *C*): This subject produced records with repeated single deflections over a range of about 60 degrees. These deflections were more prominent on the extension phase of movement. His complaints did not stress one knee, nor did his records.

In this group, the records of two patients differed from normal, and clinically there was no question of the diagnosis of osteoarthritis. In the re-

maining subject, the record was noiseless, a finding which was subsequently confirmed by the transfer of the patient to the neuropsychiatric ward. The patient whose complaints were unilateral produced noise in the affected knee only.

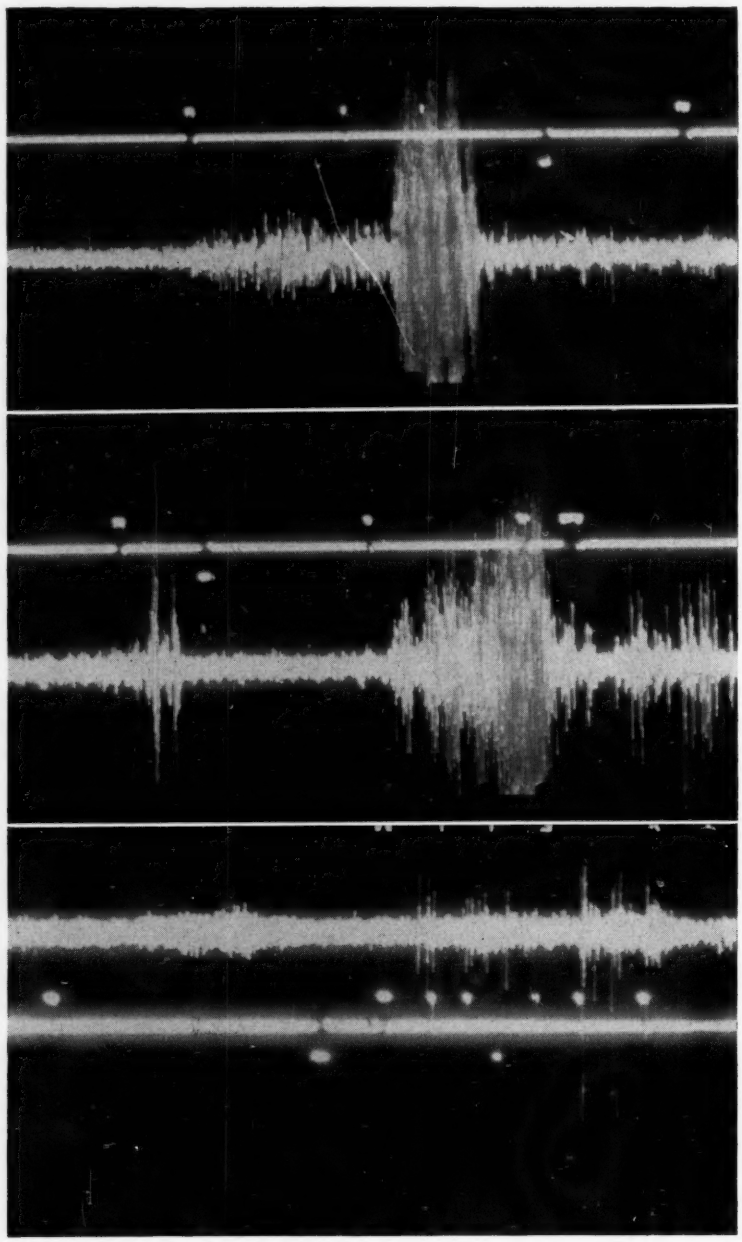


Fig. 5.—Abnormal records: *A*, Record from the medial side of the right knee of a patient with rheumatoid arthritis during flexion. *B*, Same as *A*, but during extension. *C*, Record from the lateral side of the right knee of a patient with osteoarthritis during flexion.

3. *Rheumatoid Arthritis* (Fig. 5, *A* and *B*).—Records of six patients in varying stages of the disease were obtained.

Patient 1: This patient produced noise over a range of about 10 degrees of flexion. The noise intensity was approximately 10 times that of normal subjects. The deflection appeared mainly on the medial and lateral sides of his right knee, although the patient did not complain of more discomfort in this joint. Examination of the clinical history showed that the subject had had effusion into the right knee almost continuously over the previous two years, although it gave no more discomfort than the other.

Patient 2: The noise recorded from the lateral aspect of the left knee of this patient was 4 to 5 times greater than normal over a range of 20 degrees. (The location of the noise corresponded well with the patient's complaint of discomfort.) Little abnormal noise was recorded from the opposite knee.

Patient 3: The noise recorded from the knee joints of this patient was of a different character and consisted of fine individual deflections separated by 1 or 2 degrees of flexion and extending over a range of about 50 degrees.

Patient 4: The knee of this patient produced deflections similar to those of Patient 3, viz., single deflections separated by 2 or 3 degrees of flexion and appearing over a range of 20 or 30 degrees. They were recorded mainly from the outer side of the right knee, whether or not the patient was carrying some or all of the body weight. The location of these abnormal deflections corresponded to the site of the patient's chief complaint, which was of ten years' duration.

Patient 5: The records obtained from this subject were indistinguishable from normal. He complained of pain in his knees, in his left hip joint, and in his abdomen. This discomfort made the movements of knee bending very painful and extremely slow.

Patient 6: The records of this patient showed no definite abnormality. He complained of mild generalized pain in his joints, less in his knees than elsewhere. He was discharged from the hospital the day on which joint sounds were recorded, clinically judged to be recovered.

In this group, a qualitative correlation has been found between the disease of a joint and the noise it frequently produces during movement. These joints which caused the patients most discomfort also produced most noise.

4. *Subacute Rheumatic Arthritis (In Remission).*—This patient produced records only very slightly different from normal, some noise appearing on the lateral side of the right knee.

5. *Traumatic Arthritis (Recovered Clinically).*—One patient had a history of a torn and repaired medial collateral ligament of the left knee in 1945. The records for this knee were indistinguishable from normal records. Since the time of repair, the knee has remained free of symptoms. The record of the opposite knee displayed a moderate amount of noise, in confirmation of the current diagnosis of mild rheumatoid arthritis.

CONCLUSIONS

1. Noises in normal and diseased joints of adults and children have been recorded.

2. In most cases where there was no history of joint injury or disease, the knee movements were silent except for short, sharp sounds corresponding to the familiar joint crack or snap.

3. Where a diagnosis of disease or a previous injury has been established, the records usually indicate greater than normal amounts of noise.

4. Where previously injured or diseased joints were found to be free from noise, subjective sensations of pain were minimal.

5. The method described will supplement clinical examination by allowing the production of objective and permanent records. The apparatus can be made by a relatively inexpensive addition to a standard electrograph.

We are indebted to Dr. Wallace Graham and Dr. P. S. Rosen of Sunnybrook Hospital, whose co-operation made this study possible.

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CHANGES OF RELATIVE VOLUME AND VENTILATION OF THE TWO LUNGS WITH CHANGE TO THE LATERAL DECUBITUS POSITION

R. Drew Miller, M.D.,* Ward S. Fowler, M.D.,** and H. Frederic Helmholz, Jr., M.D.**
Rochester, Minn.

THE dependent or lower lung when a person is in the lateral decubitus position increases its relative share of both oxygen uptake and ventilation. This has been observed repeatedly by means of bronchospirometry.[1-6] Indirect evidence, including alterations in the area of the lungs in anteroposterior roentgenograms[7] and alterations in the expiratory reserve volume, led early observers[1, 3] to state that the functional residual capacity decreases in the dependent lung and increases in the superior lung when the person is in the lateral decubitus position. This also has been said to be the case and has been illustrated by more recent authors[5, 8] whose apparatus permitted a direct spirometric registration of the changes in pulmonary volume during the change from the supine to the lateral decubitus position. The relative position of the diaphragm in these various positions was reported as early as 1866.[9]

The increased oxygen uptake of the dependent lung was properly attributed by Björkman[1] to an increased blood flow, presumably as a result of gravitational effects. Recent authors have attributed the increased flow to the increased pressure in the pulmonary artery of the dependent lung,[4, 5] and it has been suggested also that it is the result of alterations of pulmonary vascular resistance caused by the changes in the degree of inflation of the two lungs.[10]

This paper presents certain results of bronchospirometric recordings made with the subjects in several positions. Measurements of the tidal volume and the change in functional residual capacity have permitted certain calculations of the relative turnover rates of alveolar gases. The relationships between alveolar ventilation and perfusion and certain factors influencing them are discussed.

METHODS

One healthy man and 18 patients were studied. Included in the latter group were 16 patients with varying degrees of pulmonary tuberculosis which was bilateral in 13, and two men with large unilateral pulmonary cysts. Modified Carlens bronchospirometric catheters were used in the conventional manner.[11] Recordings were made by double closed-circuit spirometry, the spirometers being filled initially with oxygen. Measurements of oxygen consumption, tidal and respiratory minute volume, and vital capacity were first obtained with the subject in the supine position. While the recording continued, the subject was

From the Mayo Clinic and Mayo Foundation, Rochester, Minn. The Mayo Foundation is a part of the Graduate School of the University of Minnesota.
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*Section of Medicine.
**Section of Physiology.

then assisted in rolling to one lateral decubitus position which was maintained for two or more minutes. Similar recordings were consecutively made thereafter in the supine, the other lateral decubitus, and, in some cases, the supine position again.

Recordings which were satisfactory for measurement of tidal volume and functional residual capacity in all three positions were obtained for 16 subjects, three of whom were studied on two different days. For three persons, only one lateral position was studied. Recordings of sufficient length and stability to measure oxygen consumption were obtained in 12 lateral positions for seven subjects. Expiratory reserve volume was measured in the lateral positions on the normal subject and two patients, and the changes on change in posture were found to correspond approximately with the previously occurring changes of functional residual capacity. Vital capacity and expiratory reserve were not measured in the lateral positions in other subjects to minimize irregularities in the recording.

Measurement of the change in functional residual capacity was made as illustrated in Fig. 1, and the relative tidal volume of the two lungs was determined during simultaneous periods from both the mechanical integrator tracing and the tidal (inspired) volume tracing.

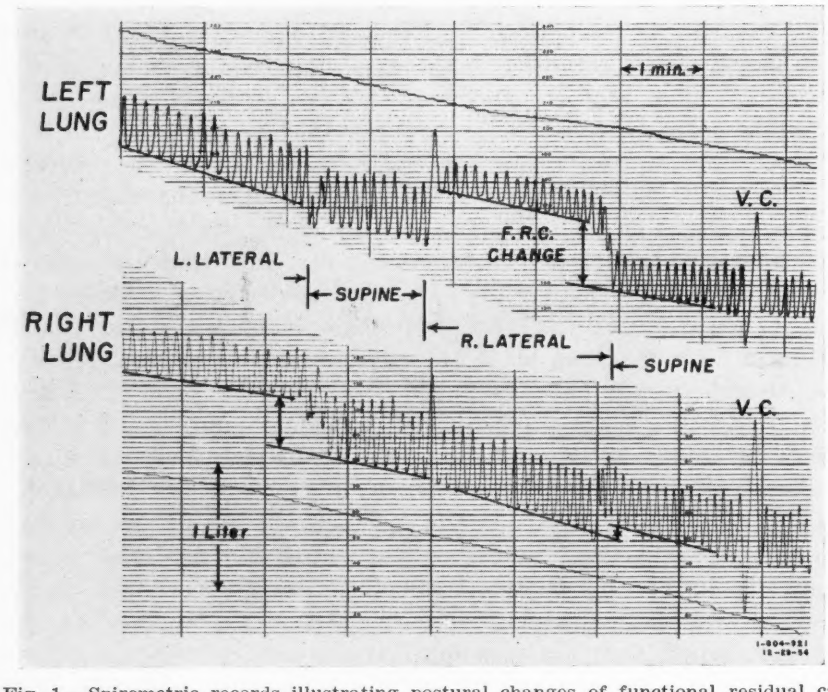

Fig. 1.—Spirometric records illustrating postural changes of functional residual capacity (F.R.C.), tidal volume, and oxygen uptake of the right and left lungs in a patient with bilateral apical tuberculosis. Records read right to left, and inspiration is upward.

Calculation of Relative Turnover Rate of Alveolar Gases.—The extent to which alveolar gases are replenished with fresh air on each inspiration can be designated by the ratio $\frac{TV - DS}{L}$

when TV = tidal volume
DS = dead space volume
L = lung volume or functional residual capacity.

With deeper breathing this ratio increases. Since the dead space is some fraction of the tidal volume, the ratio can be designated $\frac{T}{L}$

when $T = TV - DS$.

The relative depth of alveolar ventilation of the two lungs can then be stated as

$$\frac{T_L}{L_L} \Big/ \frac{T_R}{L_R} = \frac{T_L}{T_R} \times \frac{L_R}{L_L}$$

when the subscripts L and R refer to left and right lungs.

A comparison of the relative ventilation in different positions can be made if the ratios of tidal volumes (assuming a constant ratio of dead space to tidal volume) and the changes in L_R and L_L are known. For example, in a supine subject the ratio of tidal volume of the left lung to that of the right was 51/49 and respective unknown values are those of the functional residual capacity of the two lungs (L_L and L_R). In the various positions, the changes of tidal volume and functional residual capacity listed below were:

	Supine		Left Lateral		Right Lateral	
$\frac{T_L}{T_R} \times \frac{L_R}{L_L}$	$\frac{51}{49}$	$\times \frac{L_R}{L_L}$	$\frac{62}{38}$	$\times \frac{L_R + 320}{L_L - 80}$	$\frac{47}{53}$	$\times \frac{L_R - 60}{L_L + 420}$

It is apparent that the value of the product is greater for the left lateral position than for the supine or right lateral; that is, the extent or "depth" of alveolar ventilation of the left lung progressively increases in proportion to that of the right lung when the patient goes from the right lateral to supine or from supine to left lateral position. There are, of course, possible combinations of numerical values in the two terms of the expression which, with L_R and L_L unknown, make comparisons of the product indeterminate.

RESULTS

For brevity of reporting results, we shall often use the terms "dependent lung" and "superior lung" for the lower and upper lung, respectively, when the patient is in a lateral decubitus position and "supine lung or lungs" when the patient is in the supine position. On the same basis we shall also refer to a lung as being in the dependent, superior, or supine position.

Tidal Volume.—In 31 of 41 observations, the dependent lung increased its proportion of the total tidal volume over that observed in the same lung in the supine position. Increases up to 17 per cent (52 per cent supine to 69 per cent lateral) were observed. However, the extent of increase was not correlated with extent of disease judged roentgenologically or with the proportion of total tidal volume contributed in the supine position. Increases of 4 to 16 per cent of the total were noted in four lungs in which upper lobectomies had been performed previously. In all but one of 18 patients, the dependent lungs had a greater proportion of the tidal volume than the same lungs in the superior position.

Change in Functional Residual Capacity.—In all of 41 observations, the functional residual capacity of a lung increased when its position was changed from the supine to the superior, regardless of the extent of disease. The change of volume was usually several hundred milliliters, varying from 120 to 1,080 ml. The least change occurred in the left lung of a small woman who had been treated for three years by pneumothorax, and the greatest change occurred in an apparently normal lung of a large man with contralateral pneumatoceles. Increases of 300 to 600 ml. were observed in three lungs from which the upper lobes had been removed.

When the position of a lung was changed from the supine to the dependent position, the functional residual capacity of that lung usually decreased by 50 to 200 ml., although increases of about 100 ml. were observed in 4 of 41

observations, of which 3 were on the same subject. No change was observed in 9 instances. In every case the change was smaller than the increase observed in the same lung when the position of the lung was changed from the supine to the superior. In all changes to a lateral position, the increase of functional residual capacity of the superior lung exceeded any decrease of the dependent lung, giving a net increase of several hundred milliliters from that of the supine position.

Oxygen Uptake.—In 10 of 12 observations, the proportion of the total oxygen consumption increased when a lung was changed from the supine to the dependent position; no change was observed in two cases. Increases of oxygen uptake amounting to 13 to 30 per cent of the total were observed in the same lung on changes from the superior to the dependent positions. One lung, previously subjected to upper lobectomy and a five-rib thoracoplasty, almost doubled its oxygen consumption on change from the supine (18 per cent of total) to the dependent position (30 per cent).

Calculation of Relative Alveolar Ventilation.—The dependent lung increased its calculated alveolar turnover rate relative to that of the superior lung on changing from the supine to the lateral position in 15 of 20 instances for the left lung and 19 of 21 instances for the right lung. The left lung increased its turnover rate relative to that of the right lung on change of position from the right to left lateral decubitus in 14 of 18 observations. The observations which failed to show these changes were indeterminate by calculation and may or may not have done so.

COMMENT

Although the patients in this group had widely differing degrees of parenchymal disease in one or both lungs, the usual events on assumption of the lateral decubitus position were qualitatively similar to those of normal lungs. The responsible mechanisms have received scanty attention, although an understanding of them would contribute greatly to the more general problem of regional differences of function in various parts of the lungs. In the following comments we hope to enumerate, if not clarify, certain factors which require further study.

Changes in Circulation.—The relative increase in oxygen uptake of the dependent lung represents an increased alveolar blood flow, because the tensions of inspired oxygen are sufficiently high to assure practically equal arteriovenous differences for each lung, with complete saturation of hemoglobin. As mentioned before, recent investigators have suggested that the increased blood flow is the result of increased pressure in the pulmonary artery of the dependent lung[4, 5] or is due to relative decrease of pulmonary vascular resistance caused by alterations of volume of the lungs.[10] Neither explanation per se is adequate in our opinion.

The extent of alteration of oxygen uptake or alveolar blood flow was not proportional to the changes in volume. Although the total functional residual capacity of the two lungs together was not measured in the supine position,

the increase of capacity of the lung which became the superior lung on change from the supine to the lateral position was sufficiently greater than the decrease of capacity of the dependent lung to indicate a lack of direct proportionality between change of volume and change of blood flow. Three cases were observed in which the oxygen uptake of the dependent lung increased considerably (17 to 30 per cent, 28 to 37 per cent, 30 to 46 per cent) with a change of functional residual capacity of less than 40 ml. The relationship between pulmonary volume and vascular resistance has been in dispute for many years. It has been thought by some that with inflation the pulmonary vascular resistance decreases[12] and by others that it increases.[13, 14] The bronchospirometric results in man in the lateral position demonstrate that both an increase in volume and an increase in relative vascular resistance occur in the superior lung. There are, however, more attractive explanations than that pulmonary volume per se is responsible.

It may be considered that both lungs have a common pressure head between the main pulmonary artery and left atrium. However, the weight of the column of blood above a point in either the arterial or venous channels is greater in the dependent lung than in the corresponding anatomic locations in the superior lung. Since the vessels are distensible, this tends to increase their diameters in the dependent lung over those in the superior lung. The pulmonary vasculature is characterized by great distensibility, that is, an additional pressure of a few centimeters of water will increase the internal volume markedly.[15, 16] With distention of the vascular bed, the resistance to flow decreases,[13, 17] and a relative increase of flow through the dependent lung could result. However, this relatively simple situation is complicated by several other factors.

The diameter of a vessel depends on the pressures both inside and outside and also on the properties of the vessel wall. For the alveolar capillaries, the outside pressure is probably close to alveolar pressure, which varies below and above atmospheric pressure with each inspiration and expiration. For larger vessels within the lung, it is not certain what should be regarded as the pressure outside.[18] Burton[19] has pointed out that small vessels also have a critical closing pressure, determined by the size of the vessel and the active tension of the smooth muscle in the wall, such that when the internal pressure falls below a certain value, complete closure occurs. The probable importance of this phenomenon in the low-pressure, pulmonary vascular bed has been pointed out by Lilienthal and Riley.[20] Although there are large differences in internal hydrostatic pressure between dependent and superior vessels of the lungs, their relative tone, which also affects the magnitude and stability of the radius, is unknown. It seems possible that with changes of posture not only is there an average increase of flow through the dependent lung, but also a "postural" redistribution of flow may occur within each lung and lobe. Postural changes of blood flow in various parts of the lungs have been suggested by Dock,[21] and the studies of alveolar gases from upper and lower lobes of man[22] and dogs[23, 24] are consistent with relative decreases of flow in the upper lobes in

the erect posture. However, the relative blood flow to the right and left lungs is not appreciably affected on change from the supine to sitting position,[6] when gravitational effects are similar for both lungs.

Changes in Ventilation.—A redistribution of alveolar volume and ventilation between the two lungs occurs when the lateral decubitus position is assumed. The smaller end-expiratory volume of the dependent lung, with the high position of the lower hemidiaphragm, reflects gravitational effects on the mediastinal structures and intra-abdominal pressure. However, current views of thoracic mechanics do not provide a ready explanation for the increased tidal volume of the dependent lung, which has been observed even with ipsilateral diaphragmatic paralysis.[4] It is an example of the more general problem of the relationships between forces and resistances which determine the distribution of air flow to alternative pathways in the bronchial tree.

Although in the erect position different parts of the lung are normally ventilated at different turnover rates (ventilation rate per liter of alveolar gas), the location of the different areas is unknown.[25] The calculations indicate that in the lateral position the dependent lung has a greater turnover rate than the superior lung and that the differences of turnover rates between the lungs are greater than the difference in rates of alveolar ventilation, because of both decreased tidal volume and increased functional residual capacity of the superior lung. It should be emphasized that the calculations are to be regarded as probably only directionally correct, since they are based on the assumption of a constant ratio of dead space to tidal volume and uniform alveolar ventilation. However, changes of this type have been observed experimentally in dogs.[26]

Attention is directed to the distinction between alveolar turnover or dilution rate and alveolar ventilation rate for the following reasons. Valuable analyses of the factors determining the composition of alveolar and arterial gases have been developed;[27, 28] these analyses have been based on the premise that both alveolar ventilation and circulation are continuous processes. For such purposes, alveolar ventilation can be defined, as is customary, in liters per minute, and in steady states, the volume of the alveolar spaces which are being ventilated does not enter into the various mechanisms which can reduce the arterial oxygen tension below that of the mean alveolar gas. However, evidence is increasing that alveolar circulation as well as ventilation is periodic rather than continuous.[29] Thus, differences of oxygen tension between arterial blood and alveolar air are probably in part dependent on the magnitude and phase relationships of the ventilation and circulation cycles, as recognized by Riley and Cournand.[28] The magnitude of the ventilation cycle depends on the alveolar volume. It is of interest that in the dependent lung the alveolar dilution rate per breath is probably large and also the average blood flow per heartbeat is increased.

Clinical Aspects.—In clinical use of bronchospirometry, it is customary to evaluate the relative function of the two lungs with the patient at rest in the supine position. Considerable changes may occur in lateral positions, indicating that the supine measurements are not entirely representative. However,

we do not currently believe that a diseased lung which is contributing a certain fraction of oxygen uptake and ventilation in the supine position can be necessarily upgraded or downgraded on the basis of changes occurring in the lateral positions. This is based both on clinical experience with the results of resective surgery, which had been done on most of the patients in our series, and on physiologic considerations, which indicate that the relative changes which occur in the lungs when a lateral position is assumed are functions of *both* lungs rather than a reflection, primarily, of the characteristics of the single lung under particular scrutiny.

It is frequently necessary to evaluate the auscultatory breath sounds of patients who are too ill to sit up. Therefore, the lateral decubitus position is used for auscultating the posterior aspect of the thorax. A recent textbook[30] on physical diagnosis, in referring to this, stated: "Allowance must be made for the decreased ventilation of the dependent lung in interpreting the sounds heard." However these and previous measurements have shown that the ventilation of the dependent lung is relatively increased. Also the amplitude of its recorded breath sounds has been shown by Kerr[31] to be increased in normal subjects and in patients with various types of pulmonary diseases. Our auscultatory experience agrees.

SUMMARY

The changes of relative ventilation and oxygen uptake and of functional residual capacity of the two lungs that occur on changes from the supine to the lateral decubitus position have been measured bronchospirometrically in 16 patients with pulmonary tuberculosis, two with pulmonary cysts and one healthy man. The results confirm previous reports that the dependent lung when the patient is in the lateral position usually increases its relative ventilation and oxygen uptake and decreases its proportion of the total functional residual capacity. Calculations are presented which indicate qualitatively that the rate of turnover of alveolar gases is relatively greater in the dependent lung. The physiologic mechanisms responsible for the changes of alveolar blood flow and alveolar ventilation are not well understood. Clinical issues dealing with bronchospirometric examination and with auscultation of the thorax in the lateral decubitus position are noted.

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TIME FACTOR IN PRODUCTION OF THE LUPUS ERYTHEMATOSUS PHENOMENON IN VIVO

JEROME F. SICKLEY, M.D., IRVING A. FRIEDMAN, M.D.,
AND CHARLOTTE FELDHAKE, B.S.
CHICAGO, ILL.

THE recent finding[1] of the lupus erythematosus phenomenon in vivo, produced by constriction of a digit with a rubber band for 20 minutes, suggested an excellent method for the study of the time factor in the production of the L. E. phenomenon. The time factor had been studied previously in vitro by Haserick,[2] who found by the oxalated blood technique that rosettes appeared in 5 to 6 minutes and L. E. cells in 12 to 13 minutes.

MATERIAL AND METHOD

Twelve ambulatory patients with classical disseminated lupus erythematosus in whom the clot technique as well as the finger constriction technique was positive for L. E. cells were studied.

A rubber band was applied to a digit, and blood films were obtained at intervals of 5, 10, 15, and 20 minutes of constriction. These preparations were then studied for the presence of L. E. cells. Each slide was studied for a period of 10 minutes. If cells were found at 10 minutes or less, closer intervals were then observed, with films taken at 4, 6, and 8 minutes.

RESULTS

The results are summarized in Table I and illustrated in Fig. 1. L. E. cells were first found at 6 minutes in one patient and at 8 minutes in four patients. It is of interest that rosettes were seen as early as 5 minutes. Nucleophagocytosis was seen in several of the preparations.

TABLE I. TIME FACTOR IN IN VIVO PRODUCTION OF L. E. CELLS

MINUTES OF CONSTRICTION	EARLIEST APPEARANCE		
	L. E. CELLS	ROSETTES	NUCLEOPHAGOCYTOSIS
5		1	1
6	1	1	
8	4	1	
10	2	2	1
12	1		
15	3	1	
20	1		
	12	6	2

From the Hematology Laboratory and the Hektoen Institute for Medical Research of the Cook County Hospital, Chicago.
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COMMENT

The study of the time factor in the production of L. E. cells in vivo reveals that the period necessary for the production of these cells is shorter than Haserick concluded from his in vitro study; however, Haserick also found rosettes in vitro at 5 minutes.

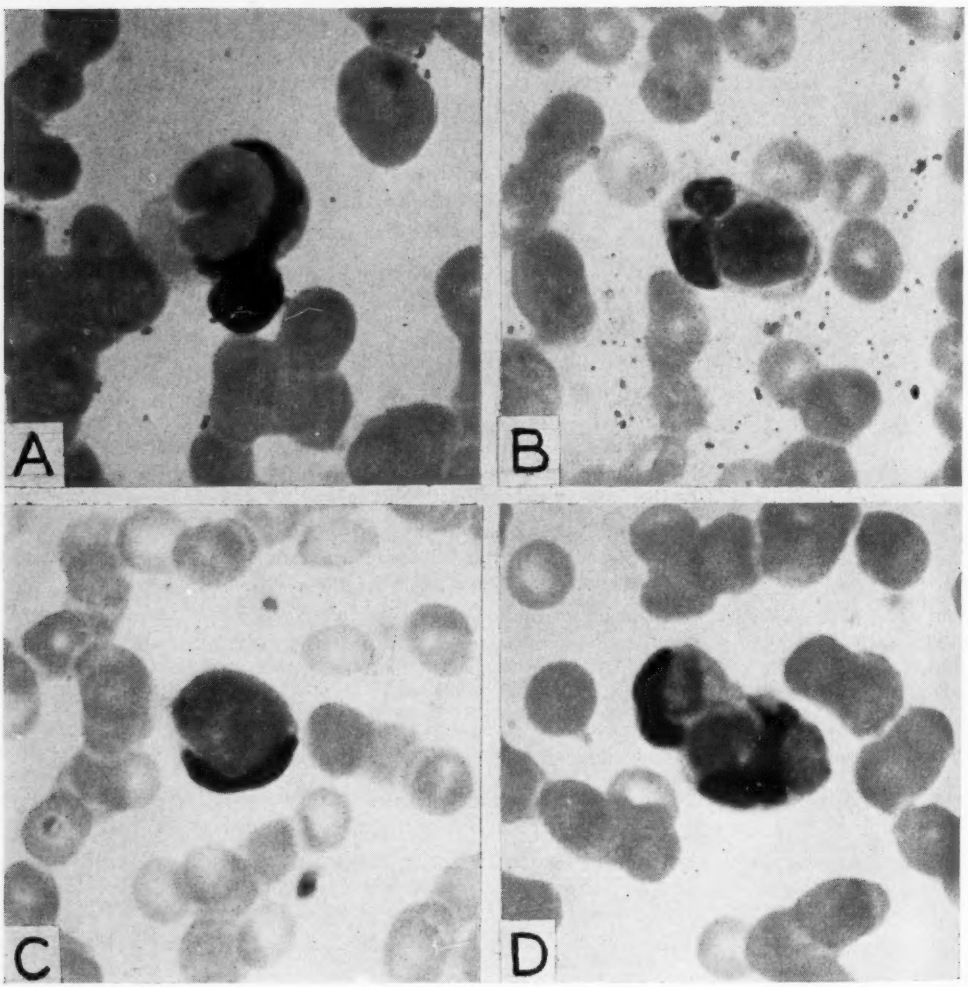

Fig. 1.—L. E. cells found at intervals of (*A*) 15, (*B*) 10, (*C*) 8, and (*D*) 6 minutes, respectively.

It is apparent from this study that the longer the constriction is applied the greater is the probability of obtaining a positive L. E. test. As a precaution, we feel that 20 minutes should be the maximum period of constriction.

The phenomenon of nucleophagocytosis appeared in two cases and is significant because the cells in nucleophagocytosis are not found in blood films obtained without constriction and may be confused with L. E. cells by the inexperienced observer.

SUMMARY

1. The time factor in the in vivo production of L. E. cells by the digit-constriction method was studied in 12 patients.

2. L. E. cells appeared as early as 6 minutes in vivo.

3. Rosettes were found at 5 minutes.

4. Nucleophagocytosis appeared in two cases, a phenomenon that does not occur in films obtained without constriction.

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LABORATORY METHODS

AN ALUMINUM HYDROXIDE-ARSENOMOLYBDIC ACID METHOD FOR THE DETERMINATION OF MINUTE QUANTITIES OF TISSUE CATECHOL AMINES

J. M. B. Bloodworth, Jr., M.D., and E. von Haam, M.D.
Columbus, Ohio

INTRODUCTION

THE catechol amines, epinephrine and norepinephrine, are produced in the adrenal medulla and sympathetic nerve endings throughout the body. However, except for the adrenal medulla, the tissue concentration of catechol amines remains exceedingly low, usually less than 1 μg per gram. Most chemical methods in current use require the extraction of many grams of tissue in order to obtain adequate quantities of catechol amines for assay. The object of the present investigation was to develop a method suitable for assay of catechol amines from small quantities of tissue.

In 1935 Whitehorn[1] published a method of catechol amine assay based on adsorption by silicic acid and measurement of the color produced by reduction of arsenomolybdic acid. In 1938 Shaw[2] modified this method and introduced adsorption on aluminum hydroxide for the separation and concentration of catechol amines. The method was further modified and adapted to the spectrophotometer by Raab[3] in 1941.

Aluminum hydroxide has remained the adsorbent of choice and is used in all methods in current use. Unfortunately aluminum hydroxide adsorbs other reducing substances from the tissue extract. Bloor and Bullen[4] demonstrated that alkaline incubation would destroy the reducing properties of catechol amines. Therefore, they suggested that the difference between the color before and after alkaline incubation of the final extract represented the true catechol amine content. We have modified this principle and applied it to the assay of tissue extracts.

METHOD

Reagents.—

1. Trichloracetic acid: 10 per cent (reagent grade).
2. Sodium hydroxide: 10 N, 2 N, 0.5 N, 2.8 N.
3. Sulfuric acid: 2.8 N.
4. *Sulfuric acid reagent:* 85.7 ml. concentrated H_2SO_4 plus 14.3 ml. distilled water.
5. *Aluminum hydroxide reagent:* Dissolve 25 Gm. of C. P. potassium alum with heating in 200 ml. of distilled water and cool to 20° C. Dissolve 5 Gm. of C. P. NaOH in

From the Department of Pathology, College of Medicine, Ohio State University, Columbus, Ohio.
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20 ml. of distilled water and add slowly to the potassium alum solution, with constant stirring by a mechanical stirrer. Filter the solution and wash the precipitate three times by resuspension with mechanical stirring in 200 ml. of distilled water. Suspend the washed precipitate in 100 ml. of distilled water. Adjust the pH to 7.5, with constant mechanical stirring, by the addition of 2 N NaOH. After one week again check the pH and readjust if necessary. Then allow the reagent to "age" for an additional week before use.

6. *Arsenomolybdic acid reagent:* Dissolve 60 Gm. of crystalline sodium molybdate and 10 Gm. of sodium arsenite in 250 ml. of water. Filter the solution and wash the filter paper several times. Add the washings to the filtrate. Then add 5 ml. of a saturated solution of bromide water to the filtrate and washings and make them up to 500 ml. with distilled water. This stock solution is stable for several months. Prepare the *arsenomolybdic acid reagent* immediately before use by the addition of 8 ml. of concentrated H_2SO_4 to 100 ml. of the stock solution.

7. *Sodium sulfite reagent:* Dissolve 5 Gm. of anhydrous sodium sulfite in 200 ml. of distilled water.

8. Buffered saline: Buffer 0.9 per cent NaCl to a pH of 7.5 with 1/1,000 M (final concentration) phosphate buffer.

9. Epinephrine standard: Pure epinephrine,* 20 μg in 100 ml. of 10 per cent trichloracetic acid.

10. Norepinephrine standard: Pure norepinephrine,* 20 μg in 100 ml. of 10 per cent trichloracetic acid.

Procedure.—

1. (a) Blend 10 Gm. of fat-free tissue for 1 minute in 40 ml. of cold 10 per cent trichloracetic acid (TCA). Allow to stand for 30 minutes in refrigerator. Filter into a 200 ml. beaker; wash filter paper and precipitate with 10 ml. of TCA. (b) Blank: Place 50 ml. of TCA in a 200 ml. beaker. (c) Place 50 ml. of epinephrine standard in a 200 ml. beaker. (d) Place 50 ml. of norepinephrine standard in a 200 ml. beaker. Handle each beaker in a similar manner except do not prepare the tubes for alkaline incubation from the standards.

2. Bring solution in beaker to pH 7.5 using a pH meter and constant mechanical stirring. Add 10 N NaOH until the solution in the beaker reaches a pH of 1.5, 2 N to pH 4.5, and 0.5 N to pH 7.5. If the solution becomes strongly alkaline, the catechol amines will be irreversibly oxidized and a pink color will appear. This will result in a lowered recovery.

3. Add 20 ml. of *aluminum hydroxide reagent* to each beaker. Mix thoroughly and immediately pour into a 100 ml. graduated cylinder.

4. Pipette 1/10 of total volume in graduated cylinder into each of six 15 ml. centrifuge tubes. Shake suspension between each filling of the pipette.

5. Centrifuge the tubes; discard supernatant.

6. Add 3 ml. of buffered saline to the precipitate in each centrifuge tube. Shake until precipitate is thoroughly suspended. Centrifuge and then discard the supernatant.

7. Divide each set of 6 tubes into 3 acid tubes and 3 alkaline tubes. The alkaline tubes need not be prepared from the standards. (a) To each alkaline tube add 2 ml. of 2.8 N NaOH; (b) to each of the acid tubes add 4 ml. of distilled water; (c) place a stirring rod in all tubes and mix well.

8. Incubate all tubes for five minutes in a boiling water bath; place immediately in a cold water bath and add 2 ml. of 2.8 N H_2SO_4 to each alkaline tube. Stir quickly with a vertical motion. This neutralization will produce a heavy white precipitate in the alkaline tubes. For the remainder of the procedure, handle all tubes (acid and alkaline tubes) in a similar manner.

9. Prepare a second set of calibrated 15 ml. centrifuge tubes labeled in a similar manner to the tubes previously prepared. Place 1 ml. of freshly acidified *arsenomolybdic acid reagent* in all tubes of the second set. The use of two sets of tubes is cumbersome but necessary to obtain the rapid and complete mixing required for consistent results.

*Supplied by Dr. M. L. Tainter, Director, Sterling Winthrop Research Institute, as L-epinephrine bitartrate and L-norepinephrine bitartrate.

10. Incubate the second tube containing arsenomolybdic acid in a boiling water bath for 5 minutes, and at the end of this time add 1 ml. of *sodium sulfite reagent* and 1 ml. of *sulfuric acid reagent* to the initial tube, stir well, and immediately pour the contents of the initial tube into the corresponding tube of the second set. Allow the initial tube to drain for 5 seconds; then transfer the stirring rod from the initial tube to the second tube and stir with a vertical motion. The heated arsenomolybdic acid and the contents of the initial tube must be rapidly and thoroughly mixed. Continue to incubate the second tube for 5 minutes, stirring each minute.

11. Cool the second tube to room temperature, dilute to 12 ml. with distilled water, and read the optical density at 535 mμ on spectrophotometer set at zero against the blank. Use acid blank for acid tubes, alkaline blank for alkaline tubes.

Calculate the 80 per cent value of the average optical density for each set of three alkaline tubes. Calculate the average optical density of the three acid tubes. The difference between the optical density of the corresponding acid and the corrected optical density of the alkaline tubes will represent the color due to catechol amines. The optical density difference can be converted to micrograms per gram of tissue by dividing by the optical density of the standard. This is possible since each standard tube represents the color due to 1 μg of epinephrine and each unknown tube represents 1 Gm. of tissue.

EXPERIMENTAL

Certain changes were made in the methods of Shaw, Bloor and Bullen, and Raab after a study of the factors involved in the color development.

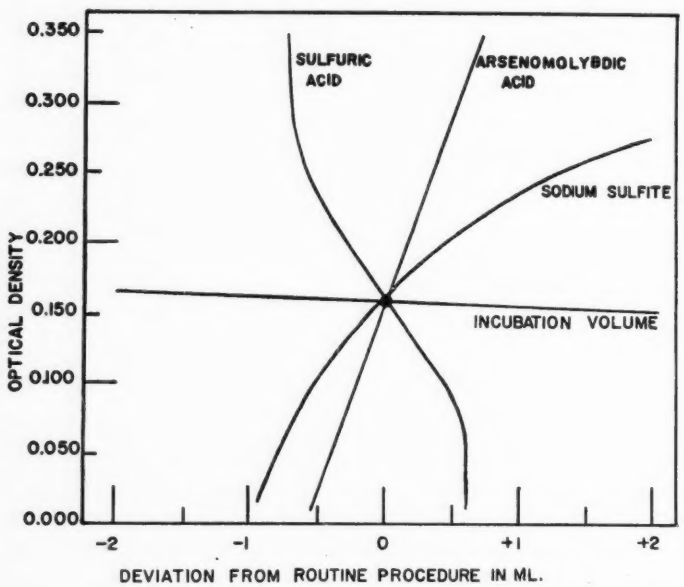

Fig. 1.—The effect of quantitative variation of different reagents on the color produced by 1 μg of epinephrine.

Fig. 1 shows that the color production is most sensitive to changes in the volume of arsenomolybdic acid but that the sodium sulfite volume and the pH are also critical variables. For this reason we have increased the total assay volume so that the measured quantities of the critical materials could be increased. It was found that acidification of sodium sulfite before addition to the sample was undesirable because of the instability of the sulfurous acid as well as the caustic nature of the material.

The recovery curves for epinephrine, norepinephrine, and heart extract plus epinephrine from 0 to 1.5 μg at 535 mμ are shown in Fig. 2. In this figure 100 per cent recovery is the optical density obtained by adding epinephrine directly to arsenomolybdic acid reagent. The lower curves show the result of the complete assay performed on various concentrations of epinephrine and norepinephrine standards. The upper curves represent the assay of different quantities of epinephrine added to a previously assayed TCA heart muscle extract. The epinephrine curves are parallel and show the upper curve displaced by a distance equal to the optical density of the previously assayed heart muscle extract. Both curves demonstrate a 90 per cent recovery of added epinephrine. Pure norepinephrine also gives a straight-line recovery curve. However, the color produced by an equivalent amount of norepinephrine is less than that produced by epinephrine. This factor must be taken into consideration if other evidence suggests a variation in the epinephrine per norepinephrine ratio.

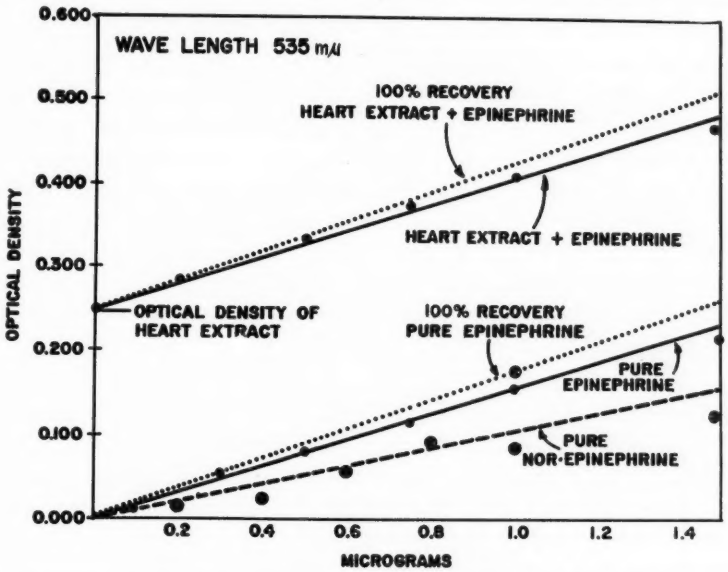

Fig. 2.—Recovery curves for epinephrine, norepinephrine, and heart extract plus epinephrine.

Studies by von Euler[5] and in this laboratory have shown that the maximum adsorption of catechol amines on $Al(OH)_3$, occurs at pH 7.5. We have adjusted our method so that the final adsorption takes place at this pH rather than pH 8.5, as advocated by Shaw and Raab. Shaw demonstrated that many extraneous reducing substances were best adsorbed on $Al(OH)_3$ at pH 3.5 to 4, while catechol amines were not appreciably adsorbed until the pH reached 7. Both Shaw and Raab performed a preliminary $Al(OH)_3$ adsorption at pH 4. We have eliminated this step because of the loss of catechol amines by partial adsorption at this pH and failure of this step to remove more than a minor portion of the nonspecific reducing substances. We have obtained essentially the same result by bringing the pH of the sample to 7.5 before addition of the $Al(OH)_3$. Alkaline incubation is still necessary to differentiate the remaining extraneous material from the true catechol amines.

Shaw suggested that the aluminum hydroxide reagent should be prepared fresh every three weeks. Our data show that the quantity of extraneous chromogens adsorbed from a tissue extract by aluminum hydroxide increases for several days after preparation of the aluminum hydroxide reagent, then gradually diminishes, and becomes constant after 5 to 7 days. Consistent results are obtained if the reagent is allowed to "age" for two weeks before use. Of course, the danger of variable adsorption from day to day is eliminated by running a daily standard, which in our experience has been found necessary. Shaw and Raab also commented on the necessity for running a daily standard.

Bloor and Bullen found that the alkaline incubation not only destroyed the catechol amines but also increased the color produced by the nonspecific chromogens of the blood. He estimated the increase at 10 per cent. Our studies on tissue extracts shown by other methods to be free of catechol amines indicate that the increase in color due to alkaline incubation of the extraneous chromogens from a tissue extract is nearer 20 per cent. We have therefore corrected the reading of the alkaline incubation tube whenever it contains a tissue extract. The correction consists of multiplication by a factor of 0.80. When the 0.80 factor is used, our heart catechol amine assays are comparable to those found by bioassay. Our adoption of this 0.80 factor has been justified by the consistency of the results.

Goldenberg[6] pointed out that the tissue must be kept at a temperature of 0° C. until acidified in order to prevent appreciable loss of catechol amines. Before handling, we have routinely placed tissue for assay in the Deepfreeze for several hours until it was solid. After freezing, the tissue to be assayed is separated, sliced into thin slices, and immediately placed in chilled TCA. Small quantities of lipid do not interfere with the assay, but excess lipid should be avoided.

Animal Recovery Experiments.—Pure epinephrine and pure norepinephrine dissolved in distilled water immediately before use were injected subcutaneously or into the ear vein of young adult rabbits. The animals were sacrificed at various time intervals by a blow on the head followed in 1 to 2 seconds by the removal of the heart. The myocardium was separated from the remainder of the heart and each specimen individually assayed.

TABLE I. THE EFFECT OF EPINEPHRINE AND NOREPINEPHRINE ADMINISTRATION ON THE CATECHOL AMINE CONTENT OF THE RABBIT HEART

	PROCEDURE	NO. OF ANIMALS	MEAN (μG/GM.)	TIME*
Controls		14	1.71 ± 0.19	
Epinephrine	2 mg. I.V.	2	12.76	5 min.
		1	14.00	10 min.
		1	8.37	20 min.
		2	8.96	30 min.
Epinephrine	1 mg. I.V.	1	2.62	10 min.
		1	2.16	30 min.
Epinephrine	0.5 mg. I.V.	1	1.95	10 min.
		1	1.25	30 min.
Epinephrine	4 mg. S.C.	2	4.80	4 hr.
	2 mg. S.C.	2	4.22	4 hr.
	1 mg. S.C.	2	1.26	2 hr.
Norepinephrine	1 mg. I.V.	2	2.41	30 min.
Norepinephrine	2 mg. S.C.	2	3.20	1 hr.

*Interval between administration of catechol amine and removal of heart.

Table I shows the result of the assays. The number of assays is insufficient for quantitative studies of dose and time relationships; however, the studies do show that the method reflects the expected increase in heart catechol amine content following the injection of epinephrine and norepinephrine. The highest values were found after intravenous injection. There was a gradual decline with time. Subcutaneous injection produced a less striking but more sustained increase in the heart catechol amine content.

DISCUSSION

Meticulous care must be given detail in this procedure, but after the method is mastered, a technician can assay 7 to 10 samples in one working day. The main limiting factor is availability of centrifuge space. Twelve tubes are required for blank and standard, 6 tubes for each sample. Thus 10 samples would require 72 tubes. For efficiency, the centrifuge should hold at least thirty-six 15 ml. tubes. Although 10 Gm. of tissue are recommended for each sample, specimens can be accurately assayed in triplicate with as little as 2 to 3 Gm. of tissue. This factor has permitted the use of this method for assay of individual organs from small laboratory animals.

This procedure is useful as a survey method in the assay of small quantities of catechol amines. Although it does not differentiate between epinephrine and norepinephrine, it is useful when a large number of assays must be done or when the available quantity of tissue is small.

SUMMARY

A method for the extraction from as little as 2 Gm. of tissue and the purification of catechol amines by adsorption on aluminum hydroxide has been combined with photometric assay. The difference between the color produced by reduction of arsenomolybdic acid with and without alkaline incubation is a measure of true tissue catechol amines. Animal experiments have shown that this method adequately assays epinephrine and norepinephrine given intravenously or intramuscularly.

We are indebted to Mr. Robert Blythe for technical assistance.

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ADDENDUM

In a recent paper, von Euler and Orwen[7] advocated the use of aluminum oxide as an adsorbent.

A SIMPLE COLORIMETRIC METHOD TO DETERMINE SUGAR IN URINE

FRANCISCO MOYA, PH.D., AND JOAN DEWAR, R.T.
HALIFAX, NOVA SCOTIA

IN A series of routine urine sugar determinations done on a group of student nurses, a number of specimens gave results ranging from a trace reaction to 1+ with Benedict's qualitative method.[1] Since the positive specimens came from apparently healthy subjects, they were further tested with Clinitest Tablets,* and a quantitative glucose determination was carried out according to the method of Nelson.[2] All specimens gave negative results by Clinitest Tablets and had glucose concentrations of less than 0.20 Gm. per cent by Nelson's method.

Looking through the literature we found that others[3, 4] have reported that Benedict's qualitative test is more sensitive than Clinitest, a property not regarded as a virtue by some authors,[4] since urine specimens from healthy subjects may give trace reactions with this test.

On close examination it was noticed that the precipitate obtained when Benedict's test was performed on the aberrant specimens, although the usual yellow-orange color, was somewhat more flocculent than cuprous oxide. The precipitate was found to contain urate when tested qualitatively with the aid of Brown's method.[5] Its high solubility in water clearly distinguishes it from cuprous oxide. The formation of the abnormal precipitate is not accompanied by a decrease in the optical density of the reagent, indicating that little or no cupric ion is removed from solution.

On the basis of these observations, a test for the quantitative or qualitative estimation of urinary sugar has been devised. Urine is treated with Benedict's qualitative reagent in Folin-Wu sugar tubes, and the remaining blue color, due to cupric ion, is measured photometrically.

METHODS

Photometric measurements were made with the aid of a Coleman Junior spectrophotometer. In Benedict's qualitative procedure, the reaction mixture was heated for five minutes in a briskly boiling water bath. Benedict's quantitative procedure[1] was carried out in large test tubes using one-fifth the amount of all reagents. In the preparation of Benedict's qualitative reagent used in the spectrophotometric method, the copper sulfate was accurately weighed on an analytical balance, whereas the other components were weighed to the nearest centigram. Standard glucose solutions were prepared by diluting a stock solution containing 5.0 per cent glucose in saturated benzoic acid.

From the Department of Biochemistry, Victoria General Hospital, Halifax, Nova Scotia.
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*Manufactured by Ames Company, Inc., Elkhart, Ind.

The Colorimetric Procedure Consists of the Following Steps.—

1. With the aid of a 0.2 ml. measuring pipette, 0.2 ml. urine is transferred to the bottom of a Folin-Wu sugar tube.

2. Then is added 4.0 ml. of accurately prepared Benedict's qualitative reagent.

3. The contents of the tube are mixed by gentle rocking so that the fluid does not go above the constricted segment.

4. A blank and standards are prepared by replacing the specimen with 0.2 ml. distilled water and standard solutions, respectively.

5. All tubes are heated in a briskly boiling water bath for eight minutes.

6. After cooling in a cold water bath, the tubes are filled up to the 25 ml. mark by the addition of distilled water and mixed by inversion.

7. The precipitate, if any is formed, is removed by filtration through Whatman No. 42 filter paper.

8. The intensity of the blue color of the filtrate is read in a spectrophotometer at 650 mμ, setting the instrument at 100 per cent transmittance with water. The color is stable for at least two hours and probably for much longer.

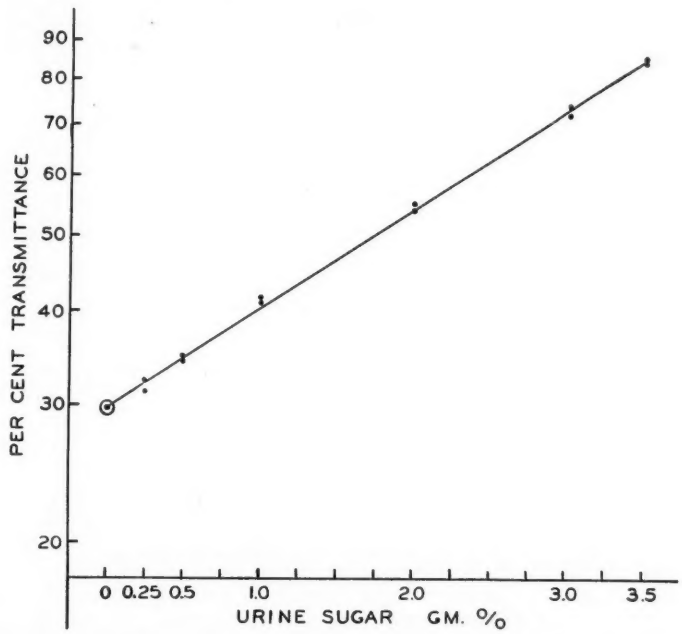

Fig. 1.—Standard curve. Glucose standards prepared from a 5.0 per cent stock solution in saturated benzoic acid.

Fig. 1 represents a standard curve obtained by the above procedure. It can be seen that a linear relationship exists up to a concentration of 3.5 Gm. per cent. This curve was found to be reproducible from day to day. It is not necessary, however, to do a standard curve with each group of unknown determinations, since it is possible to calculate an unknown concentration with the aid of the optical densities of the reagent blank and one standard. A solution containing 1 per cent glucose dissolved in saturated benzoic acid is an adequate standard.

$$C_u = C_s \times \frac{(D_b - D_u)}{(D_b - D_s)}$$

where, C_u = concentration of the unknown in Gm. per cent
C_s = concentration of the standard in Gm. per cent
D_b = optical density of the reagent blank
D_u = optical density of the unknown
D_s = optical density of the standard.

Specimens having concentrations in excess of 3.5 Gm. per cent are diluted before carrying out the determination.

RESULTS

In the course of selection of the most favorable conditions for the procedure described above, several factors were studied.

When the reaction was carried out in test tubes of various diameters, linearity was lost, particularly at concentrations below 1.0 Gm. per cent.

Fig. 2 shows that six minutes is the minimum time required to complete the reaction between glucose and the reagent. No change in optical density was observed if the boiling period was increased to ten minutes. We have arbitrarily selected a heating period of eight minutes to coincide with that of Folin's blood sugar method,[6, 7] the photometric adaptation of which is used routinely in this laboratory.

Ordinary filter paper such as Whatman No. 2 does not completely retain the cuprous oxide precipitate; however, clear filtrates are obtained with paper of greater retentiveness (Whatman No. 42).

The choice of wave length for photometric reading was based on the findings presented in Fig. 3, where the optical density of supernatants is plotted against the wave length. It can be seen from Curve A that at 650 mμ the absorption of the reagent blank is high. The decrease in absorption due to the presence of glucose is given by Curve B and is approximately maximal at 650 mμ. Curve C represents the absorption spectrum of a supernatant obtained from an aberrant urine specimen which was positive by Benedict's qualitative test and negative by Clinitest. It should be noted that the absorption of this last curve at 650 mμ is essentially the same as that of the blank (Curve A); this shows that little or no cupric ion has been removed from the solution. Absorption at wave lengths lower than 500 mμ does not seem to be markedly influenced by the presence of glucose. Urine specimens which were negative by all procedures or which contained different amounts of sugar had absorptions ranging at random in the area contained by Curves B and C.

TABLE I. RECOVERY OF ADDED GLUCOSE BY COLORIMETRIC METHOD

GLUCOSE			
PRESENT (Gm. per 100 ml.)	ADDED (Gm. per 100 ml.)	TOTAL (Gm. per 100 ml.)	RECOVERED (Gm. per 100 ml.)
0.36	0.50	0.87	0.51
0.37	0.50	0.87	0.50

Table I shows a representative recovery test in which enough glucose was added to increase the sugar concentration of the unknown by 0.50 Gm. per cent. As a rule, recoveries ranged between 100 and 105 per cent of theory, even when the concentrations tested were as low as the ones shown in Table I.

The colorimetric method was compared to Nelson's method and to Benedict's quantitative procedure. Thirty-two specimens of urine known to contain sugar were analyzed by all 3 procedures. To do determinations by Nelson's method, the urine was diluted and then treated with deproteinizing reagents.

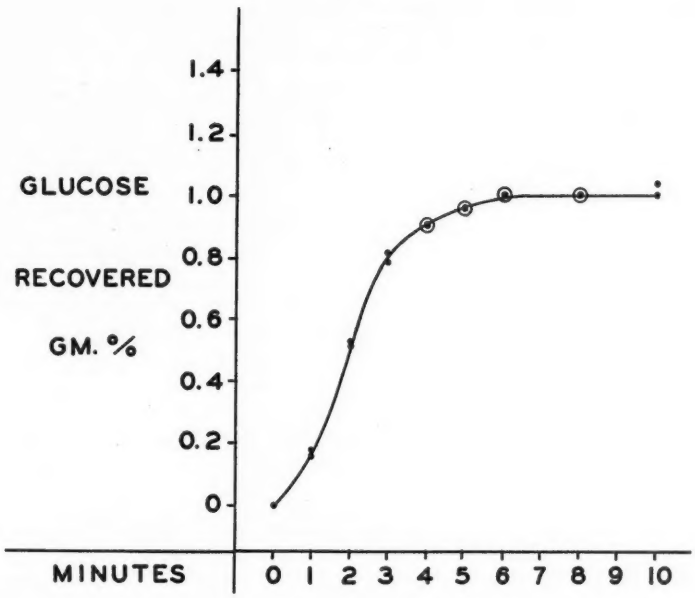

Fig. 2.—Relation of boiling time to amount of standard recovered. Glucose determinations, with their respective blanks, were carried out in duplicate on aliquots of a 1 per cent standard solution heating for the periods of time indicated on the abscissa. All results were read from a standard curve prepared in the usual manner.

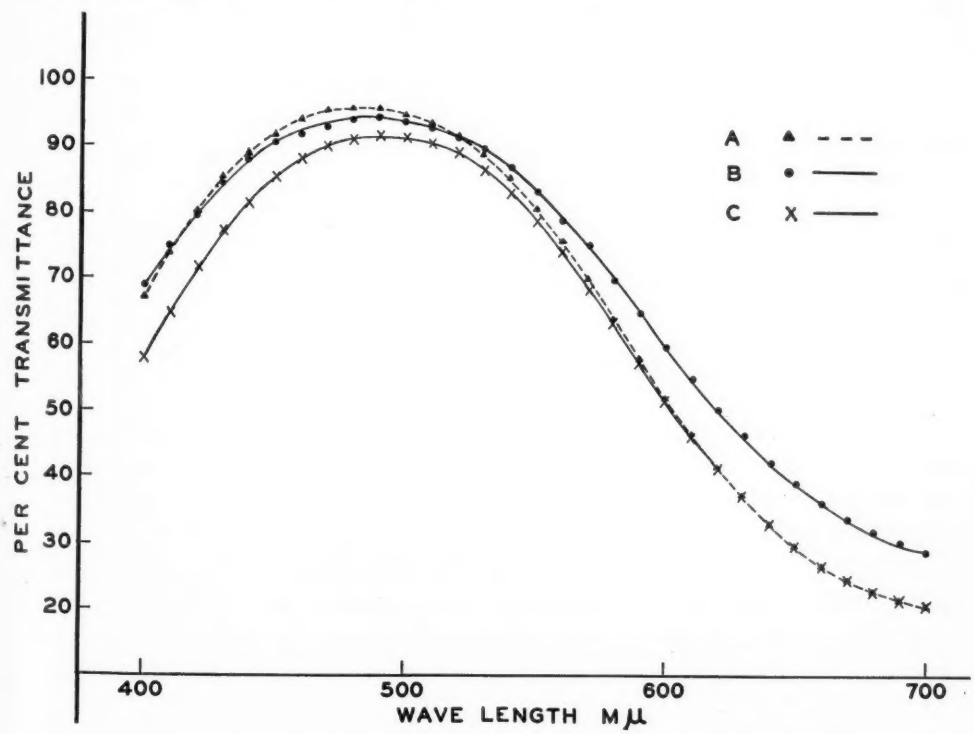

Fig. 3.—Transmittance-wave length curves done on supernatants obtained by quantitative procedure described. Curve A, Reagent blank: 0.2 ml. water. Curve B, Positive urine: 0.2 ml. urine containing 1 Gm. per cent sugar. Curve C, False positive urine: 0.2 ml. urine negative by Clinitest and 1+ by Benedict's qualitative.

TABLE II. COMPARISON OF THE COLORIMETRIC AND BENEDICT'S QUANTITATIVE PROCEDURES TO NELSON'S METHOD

RANGE OF SUGAR CONCENTRATION	NO. OF DETERMINATIONS	AVERAGE DIFFERENCE FROM NELSON'S METHOD IN GM. %	
		COLORIMETRIC	BENEDICT'S
0 – 0.5	5	0.14 (–0.09 to –0.22)*	0.07 (–0.01 to 0.15)*
0.5 – 1.0	7	0.19 (–0.06 to –0.30)	0.07 (–0.04 to 0.12)
1.0 – 2.0	10	0.23 (–0.13 to –0.29)	0.09 (–0.15 to 0.15)
2.0 – 10.0	10	0.18 (–0.03 to –0.27)	0.06 (–0.06 to 0.18)

*Figures in brackets give range of differences.

In Table II the specimens have been grouped according to the glucose concentration found by Nelson's method. In calculation of the average deviations from the values found with this procedure, the differences were added irrespective of their algebraic sign. Results obtained with Nelson's procedure were consistently somewhat higher than those found by the colorimetric method and usually, though not always, higher than those obtained by Benedict's quantitative procedure. The average differences between the Nelson method and the two urinary procedures were 0.1 and 0.2 Gm. per cent for Benedict's and the colorimetric methods, respectively.

DISCUSSION

It appears from our observations that the high sensitivity ascribed to Benedict's qualitative test[3, 4] may be the result of a false positive reaction. The yellow precipitate that forms in the absence of a correspondingly high glucose concentration has the same color as the usual cuprous oxide, and if left standing for some time, it settles to the bottom of the tube and becomes almost indistinguishable from a true positive result. On shaking, however, it is possible to notice that it is more flocculent than cuprous oxide.

The quantitative colorimetric method described above can also serve as a qualitative test. A false positive reaction will be easily detected, since the formation of a yellow precipitate will not result in a decrease in the optical density of the blue supernatant.

The colorimetric procedure described above agrees with Nelson's method almost as well as Benedict's quantitative procedure, the differences usually being of the order of 0.2 and 0.1 Gm. per cent, respectively. For most clinical purposes, however, this degree of precision is quite satisfactory. In addition, the colorimetric procedure has several advantages: It lends itself well to determinations in series and, provided care is taken in measuring the small volume of specimen used, no specialized skills are necessary. Benedict's quantitative method requires experience and considerable care. If the sugar concentration is low, relatively large volumes of urine are needed to titrate the reagent with resultant accumulation of urinary pigments which tend to mask the end point. Conversely, when the concentration of sugar is high, it is necessary to dilute the specimen in order to accurately titrate the reagent. The colorimetric method, on the other hand, presents a linear relationship up to a

sugar concentration of 3.50 Gm. per cent. Of 699 quantitative sugar determinations done in our laboratory, only 44 exceeded the upper limit of the colorimetric method.

The simplest procedure to detect sugar in urine is by Clinitest Tablets, which method, unlike Benedict's qualitative test, does not give false positive results. However, we have frequently found the tablets to disagree with Benedict's quantitative method, even when both tests were performed by experienced and skilled technicians. We have also found that replicate determinations on the same specimen with the use of fresh tablets from the same container could vary, for instance, from 2+ to 3+. Our experience has convinced us that their use should be limited to the qualitative detection of sugar only with the qualification of "high," "medium," or "low." The grading of the results according to the color chart provided by the manufacturer exceeds both the accuracy and the precision of the method. It may be that the high humidity in Halifax adversely affects the tablets, but no evidence of deterioration (blue coloration) has been observed.

SUMMARY

1. Evidence is presented indicating that what may appear as high sensitivity in Benedict's qualitative procedure may be due to false positive reactions.

2. A simple quantitative procedure to determine urinary sugar is described.

3. The method consists of treating urine with Benedict's qualitative reagent in Folin-Wu tubes and measuring photometrically the remaining cupric ion.

4. The average difference between this procedure and Nelson's method is 0.20 Gm. per cent.

5. The colorimetric procedure can also serve as an improved qualitative test.

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THE PROCONVERTIN TEST: A SIMPLIFIED METHOD AND ITS APPLICATION TO THE STUDY OF ANTICOAGULANT PROCESSES

DIONYSIOS ADAMIS, M.D., HERBERT S. SISE, M.D., AND DELBERT M. KIMBALL, A.B.
BOSTON, MASS.

THE mode of action of proconvertin in the mechanism of coagulation is well known from the original works of Owren,[2, 3] Alexander and associates,[1] and Koller and co-workers.[4] For its quantitative estimation, a simple test requiring plasma artificially rendered free of proconvertin is used. The difficulty of rendering plasma free of proconvertin without undue loss of prothrombin is a consequence of the similarity of their physiocochemical properties. Owren[5] used Seitz filter pads containing 20 per cent asbestos. Duckert and associates[6] separated prothrombin and proconvertin by means of column chromatography. The present paper describes a simplified method for adsorbing of proconvertin from beef plasma by filtration through powdered wood charcoal.

METHODS AND MATERIALS

The method of Owren and Aas[5] for the determination of proconvertin was followed except for the preparation of proconvertin-free plasma and the use of commercial dried rabbit brain thromboplastin instead of human brain thromboplastin. The technique consists of measuring the clotting time of the following mixture: 0.2 ml. proconvertin-free beef plasma; 0.2 ml. of a 1:10 dilution of the test plasma in diluting solution; 0.2 ml. of commercial dried rabbit brain thromboplastin (Difco); 0.2 ml. of $CaCl_2$ 0.03 M.

Proconvertin-free beef plasma was prepared by filtration of bovine plasma through powdered wood charcoal rather than through Seitz filter pads containing 20 per cent asbestos. It was difficult to recover sufficient prothrombin in the Seitz filtered plasma to achieve a satisfactory excess of prothrombin on which the test depends for its accuracy. Owren and Aas refer to such difficulties and suggest fortification of the filtered plasma with prothrombin made by adsorption of more filtered plasma with suitable agents and subsequent elution of prothrombin with citrate or phosphate buffer. Powdered hard-wood charcoal had a more selective adsorptive capacity for proconvertin than pads containing 20 per cent asbestos. Several types of charcoal were tried, including various forms of animal charcoals and a large number of "activated" wood charcoals. These preparations tended either to remove all clotting factors or were nonselective between prothrombin and proconvertin. After extensive trial, various makes of powdered wood charcoal were found to be satisfactory. The preparation most often used has been "L-50"*

The method evolved is as follows: Nine parts of whole bovine blood† is collected in one part potassium oxalate monohydrate, 2.5 per cent. The plasma is separated by centrifugation. In the bottom of a glass Buchner funnel,‡ 4.25 cm. in diameter, are placed five ordinary filter pads of the same diameter as the funnel. Above them are put 15 Gm.

From the Department of Medicine, Tufts University School of Medicine, and the Anticoagulant Laboratory, Boston City Hospital.

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*Made by the Springfield Facing Company, Willimansett, Mass.

†Blood from cows rather than steers in our experience is apt to contain more prothrombin.

‡With fritted disc, coarse porosity, Pyrex brand glass, capacity 60 ml., disc diameter 40 mm. (Corning).

of powdered wood charcoal and above this another filter paper or a layer of gauze to prevent admixture of charcoal with plasma. This filtering layer is pretreated by allowing 20 ml. of 0.85 per cent saline to pass through under suction in order to pack the layer of charcoal.

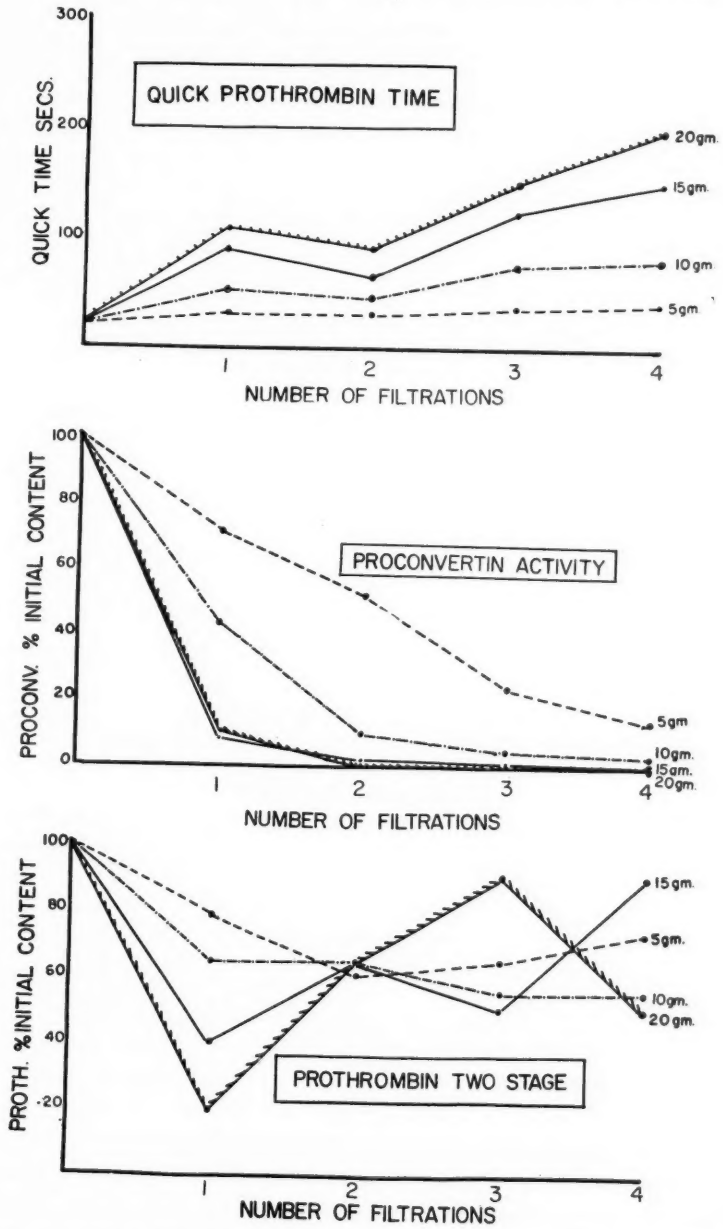

Fig. 1.—Effect of repeated filtrations of 50 ml. of bovine plasma through varying amounts of charcoal on prothrombin time, proconvertin activity, and prothrombin concentration.

Fifty milliliters of bovine plasma is then placed in the funnel but allowed to filter by gravity only. Two or three passages of the *same plasma* through the *same carbon* are sufficient to produce essentially proconvertin-free plasma. The first filtration takes 6 to 8 hours, the

second about 2 hours, and the third, 1 to 1½ hours. After the last filtration, suction is used for removal of a relatively large amount of plasma soaked in the charcoal. All procedures are done at 4° C. To determine the optimal amount of carbon, rate of filtration, and amount of plasma, each of these parameters was varied in rotation while the other two were kept constant. Two examples of this method of approach are shown in Fig. 1 and Table I. The concentration of prothrombin in the second or third filtrate frequently is higher than in the first filtrate, indicating a "washout" effect, and this is illustrated in the figure. The data indicated that 15 Gm. of charcoal was the smallest amount that would satisfactorily adsorb 50 ml. of plasma, and the slower the rate of filtration, the better the degree of separation.

Human blood was collected with ordinary glass syringes and needles, and 4.5 ml. was added to a tube containing 0.5 ml. of 0.1 M sodium oxalate. Plasma was separated by centrifuging at 4,000 r.p.m. for five minutes in a small-angle centrifuge.

Diluting solution is made according to Owren, namely: to 800 ml. of 0.85 NaCl solution, add 200 ml. of Veronal buffer and 700 mg. potassium oxalate monohydrate.

Veronal buffer is made by adding to 570 ml. of 0.1 M sodium diethylbarbiturate 430 ml. of 0.1 M HCl and 5.67 NaCl. The pH is measured and adjusted to 7.35 to 7.40.

The one-stage prothrombin time was performed by Quick's method,[7] using commercial dried rabbit brain thromboplastin. The modified two-stage method of Ware and Seegers[8] was used for determination of prothrombin. Five per cent of $BaCO_3$ beef serum and 5 per cent preparation of proconvertin from human serum by $BaSO_4$ adsorption and citrate elution were used as supplements for proaccelerin and proconvertin, respectively.

Human serum used for filtration was collected by drawing several samples of whole blood and allowing them to clot at 37° C. The blood was incubated for one hour and then oxalated (1 part sodium oxalate to 10 parts of clotted whole blood). This was incubated further for one hour and the serum drawn off, pooled, and refrigerated for twelve hours before filtering.

TABLE I.

	PROTHROMBIN % INITIAL VALUE	PROCONVERTIN % INITIAL VALUE	QUICK PROTHROMBIN TIME
60 drops per minute	60	15	45 sec.
10 to 20 drops per minute	70	0.5	3 min., 55 sec.
Gravity	60	0	18 min.

The effect of various rates of filtration of bovine plasma through charcoal on prothrombin, proconvertin, and Quick prothrombin time. The same plasma was used, and the volume of plasma and amount of charcoal were kept constant.

RESULTS

In spite of complete loss of all measurable proconvertin in the filtered sample, there is a 40 to 90 per cent recovery of the original value of prothrombin. Proaccelerin is decreased 40 to 75 per cent over the control value but not to a degree sufficient to negate use of filtered plasma as a reagent, since beef plasma normally contains many times the activity found in human plasma. Loss of fibrinogen occurs through precipitation at 4° C. Sufficient amounts remain for the purpose of the test.

To test the degree of separation of prothrombin and proconvertin, a sample plasma which had been filtered 6 times and had an indefinitely prolonged Quick prothrombin time was adsorbed with barium sulfate, 50 mg. per milliliter, which is known to adsorb both proconvertin and prothrombin quantitatively. The barium sulfate was washed carefully several times with one-half volume of normal saline, once with 0.1 M sodium oxalate and then eluted with sodium

citrate 0.1 M one-half volume and the eluate dialyzed overnight with distilled water. The prothrombin so eluted was thus theoretically free, except for possible trace amounts, of both proconvertin and proaccelerin. To test this assumption, a sample of this prothrombin was incubated with thromboplastin and calcium in the two-stage system and tested at intervals for the elaboration of thrombin. Without addition of both proconvertin (material derived from $BaSO_4$ adsorption and citrate elution of human serum) and proaccelerin ($BaCO_3$ adsorbed beef serum), there was no or very little elaboration of thrombin in fourteen minutes (Fig. 2). This clearly establishes the degree of freedom of proconvertin in filtered plasma. In doing the two-stage method for prothrombin on carbon-filtered plasma, it is essential to add proconvertin; otherwise there is incomplete conversion to thrombin, and the result is falsely low. At least 5 per cent proconvertin, as well as 1 per cent proaccelerin, in the solution used for diluting the test material is indispensable for complete conversion of prothrombin. Oxalated human serum was adsorbed in the same

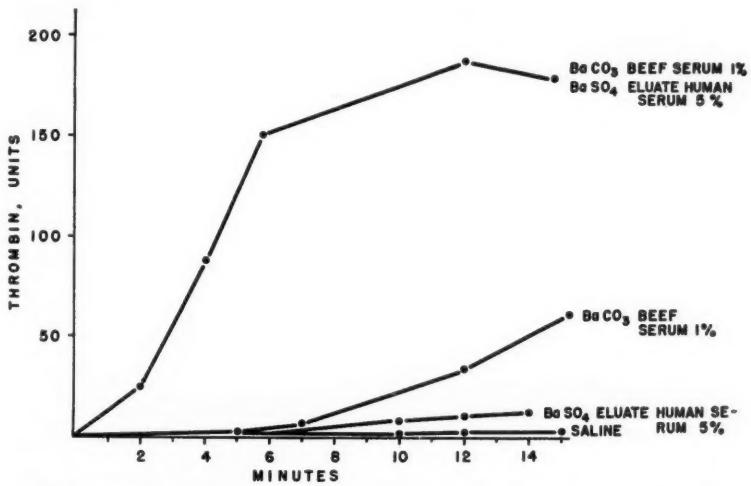

Fig. 2.—Effect of accelerators on conversion of "pure" prothrombin.

manner. The proconvertin activity before adsorption was 75 per cent, and after three filtrations through the same layer of charcoal, no proconvertin activity could be elicited.

DISCUSSION

A readily available method of assay for proconvertin has several practical clinical and academic applications. The use of the Seitz filter pads may indeed be satisfactory if the correct make of pads is employed. In our experience, only one company* supplied filter pads which gave consistently good results, and in this case there was usually only 20 to 30 per cent of the original prothrombin recovered. The variability may be responsible for the failure of others to achieve the desired results.[9] The method herein described requires

*Carlson of England.

no special equipment, results in a smaller loss of prothrombin, is reliable, and therefore makes this assay widely available for clinical and other studies.

In clinical use we have found by this assay method that a comparison of the response of prothrombin and proconvertin to the anticoagulant phenindione is of some use in the early prediction of those patients who will be sensitive or resistant to the drug.[10] About two-thirds of patients after phenindione administration showed a fall of proconvertin which was greater than the fall in prothrombin activity in the first three days. These patients required either average or higher dosage of phenindione to maintain the one-stage prothrombin time within the therapeutic range. One-third, however, showed a fall in prothrombin activity which was greater than the fall in proconvertin in the first three days. The majority of this group required only small doses of phenindione to maintain the one-stage prothrombin time within the therapeutic range.

The response of one patient,* with congenital deficiency of proconvertin, to 200 mg. of vitamin K_1 emulsion intravenously administered was observed.

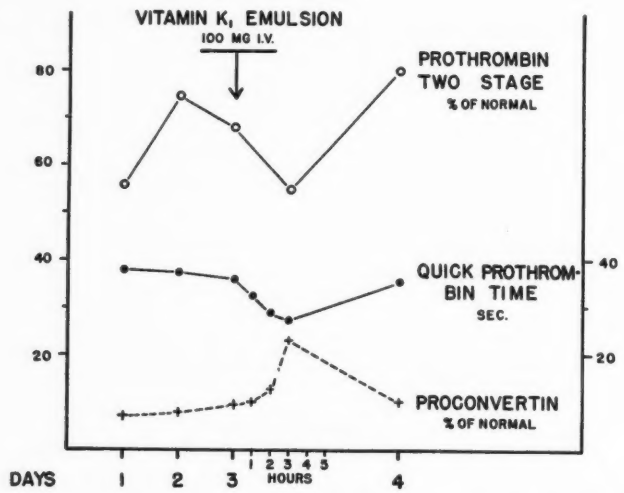

Fig. 3.—Effect of vitamin K_1 in congenital hypoproconvertinemia.

The results illustrated in Fig. 3 show a transient, small, but quite significant rise in proconvertin three hours after administration, but this change had disappeared by the next day. The change was reflected by a shortening of the one-stage prothrombin time. The fluctuations of the two-stage prothrombin assay are unexplained. The possibility of use of vitamin K_1 emulsion in bleeding episodes as a result of congenital deficiency of proconvertin is suggested.

The use of the proconvertin test in studies of the clotting mechanism may be helpful in the future, but since the consumption of proconvertin during

*We are grateful to Dr. Jane Desforges for allowing us to study this patient.

spontaneous coagulation is so small, and since the active principle convertin in small quantities shortens the clotting time in the reacting mixture, its application to fresh serum may be misleading.

The possible use of the assay as a method of measuring trace proteins in serous fluids appears to have some use. We have found surprisingly large quantities of proconvertin in various types of fluids, and we feel that its concentration may be of aid in differential diagnosis. This will be reported in a later communication.

SUMMARY

A simplified method for removing proconvertin from plasma by filtration through powdered wood charcoal has been outlined. The degree of freedom from proconvertin of prothrombin prepared from carbon-filtered plasma is demonstrated. Some applications of the test are illustrated.

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A MODIFIED RESTRAINING CAGE FOR ISOTOPIC STUDIES

WILLIAM W. BURR, JR., PH.D., JOHN A. MARCIA, B.S., AND
HERBERT C. TIDWELL, PH.D.
DALLAS, TEX.

IN ANY in vivo tracer study involving the recovery of C^{14}, one problem that must be solved is that of housing the animal in such a manner as to permit all sample collections and measurements desired. The metabolism cage designed by Roth and associates[1] or modifications of it are extremely useful when a rat is to be maintained for a period of several hours or even days during collection of the expired CO_2, and of urine and feces. However, in some studies in this laboratory it has been necessary to obtain complete recovery of respiratory CO_2 and to collect blood samples from the tail of the rat at thirty-minute intervals. With these two objectives in mind, the cage shown in Fig. 1 was developed. The inner chamber of the cage incorporates the principle of the restraining cage originated by Bollman[2] and minimizes the amount of motion permitted the animal.

Fig. 1 shows the cage both disassembled and assembled. The outside cage is made of Pyrex tubing, approximately 2½ inches in outside diameter and 8 inches in length. One end of the tube is provided with a side arm, whereas the other is closed with a 24 by 40 standard taper outside joint. The inner joint contains a small center tube for attaching a water bottle and also is equipped with a side arm for the removal of the expired air. The restraining chamber consists of two Plexiglas rings, ¼ inch in thickness, joined with 9 brass rods. The rods, ⅛ inch in diameter and 7½ inches in length, are threaded on one end. The Plexiglas rings are 2 1/16 inches in outside diameter, with center openings of ½ inch in the forward threaded ring and ¾ inch in the second, nonthreaded, ring. This second ring is provided with two set screws which permit it to be secured in place at different distances from the forward ring. This adjustable distance permits adequate accommodation for rats of various sizes. The cage was designed for animals weighing between 150 and 200 grams; for heavier rats a larger tube is recommended. The open end of the Pyrex housing is covered with rubber tissue. A small aluminum rod and Plexiglas support was constructed for the cage and is also shown in Fig. 1.

In the use of this cage, the rat is placed in the restraining compartment and the assembly inserted into the Pyrex tube. A water bottle is placed as

From the Department of Biochemistry, The University of Texas Southwestern Medical School, Dallas, Tex.

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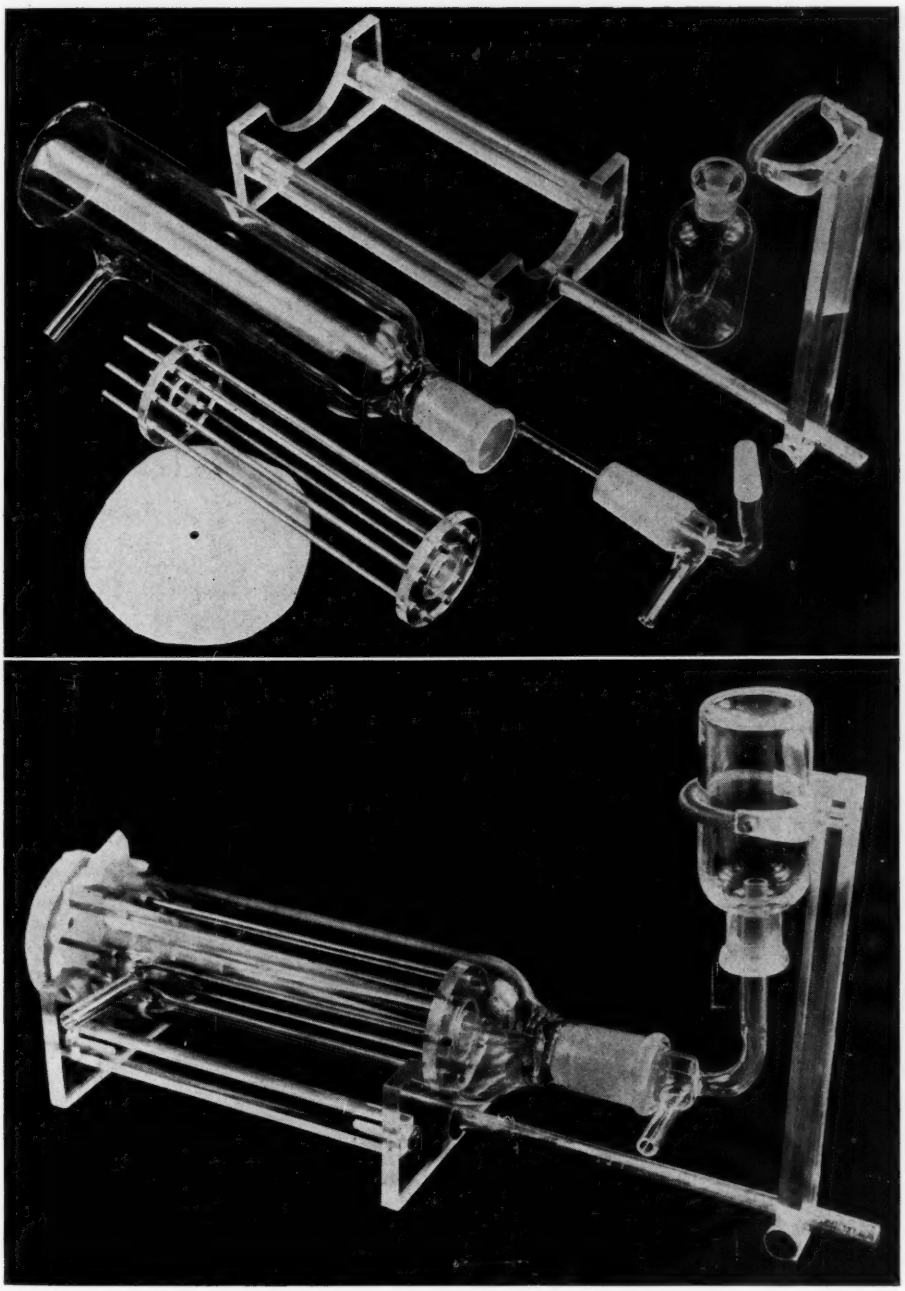

Fig. 1.—The disassembled and assembled cage.

shown in Fig. 1. A small opening is made in the rubber tissue with a No. 1 cork borer. After the insertion of the tail of the rat into this opening, the dam is tied in place on the open end of the cage. This makes the tail easily accessible for obtaining blood samples. During the experiment, air freed of CO_2 is continuously drawn into the cage through the side arm. The respiratory CO_2 leaves by way of the inner joint and is collected in 1 N NaOH in gas washing bottles. A 3-way stopcock in the line leading to two sets of traps permits the continuous collection of CO_2 with no loss during recharging of the gas washing bottles. The relatively small volume of this cage has the advantage of allowing a rapid sweeping out of the CO_2. The cage is further recommended for its simplicity and ease of construction. It has proved to be very useful in this laboratory. No provision has been made in the design for separate collection of excreta; however, a catheter could be brought out through the rubber tissue for urine collection.

SUMMARY

A modified rat restraining cage, which has proved useful in isotopic studies using C^{14}, is described.

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CULTURE MEDIA FOR SALMONELLA TYPHI AND THE EFFECT OF COMPLEMENT DESTROYING AGENTS

KENNETH C. WATSON, M.D. (ABER.)
PIETERMARITZBURG, SOUTH AFRICA

THE facility with which *Salmonella typhi* grows in a bile-containing medium has made the use of such media common in the isolation of the organism from the blood stream. Bile was first employed in this way by Conradi[1] and by Kayser.[2] In addition to a growth-promoting effect, bile-containing media have the advantage over such blood culture media as glucose broth and tryptose phosphate broth in that the bile, to a large extent, inhibits the growth of skin contaminants such as *Staphylococcus albus*. In the isolation of *S. typhi* from feces, however, it is usual to employ some enrichment medium, such as selenite F medium or tetrathionate broth. Such media prevent the overgrowth of coliforms and allow the growth of organisms of the Salmonella and Shigella groups.

As part of a study of the recovery of *S. typhi* from the blood stream, we have compared the efficacy of these various media and in addition have investigated the effect of adding various complement destroying agents.

METHODS AND RESULTS

A. *Comparison of Bile Salt Broth, Selenite F Medium, and Tetrathionate Broth.*—

1. *Procedure:* Bile salt broth was prepared by adding sodium taurocholate to digest broth to a concentration of 0.5 per cent. Selenite F medium and tetrathionate broth were prepared according to the usual formulae. Five milliliter quantities of each were placed in sterile 5″ by ⅝″ tubes in duplicate. A twelve-hour digest broth culture of a freshly isolated strain of *S. typhi* was prepared, and serial tenfold dilutions were made in 0.85 per cent saline. One milliliter amounts of each of two suitable dilutions (containing approximately 50 and 500 organisms per milliliter as estimated by plate count technique) were added to the media in each duplicate set, so that the two tubes of each pair contained approximately 8.3 and 83 organisms per milliliter. The tubes were then incubated at 37.0° C. At varying periods of incubation, 0.02 ml. amounts were spotted onto the surface of dried nutrient agar plates, and the number of colonies developed after 24 hours' incubation of the plates were counted, according to the technique of Miles and Misra.[3]

2. *Results:* The figures of the colony counts in Table I show that during the first six hours the rate of growth was more marked with bile broth medium than with the other two, and after 24 hours this difference was still apparent. The results in Table I are representative of the findings, with various batches of media and with varying strains of organisms. We have observed, however,

From the Department of Pathology, Edendale Hospital, Pietermaritzburg, South Africa.
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that with some batches of selenite F medium, the continuation of the growth study beyond 24 hours often resulted in a fall in the number of colonies between 24 and 48 hours. With a small enough initial inoculum (less than 20 organisms) complete sterility was often observed even after as short a period of incubation as six hours. In the case of tetrathionate medium, similar inhibition of growth between 24 and 48 hours was sometimes observed, particularly with batches of medium which had been allowed to stand for three or four days before use. Inhibition, however, was never as marked as in the case of the selenite F medium. The findings seemed to indicate quite definitely that bile broth medium is the medium of choice in the cultivation of *S. typhi.*

B. *The Effect of Complement-destroying Agents in the Isolation of S. Typhi From Whole Blood.*—

1. *Procedure:* In this study we used 0.5 per cent bile salt broth as the culture medium. The effect of the following complement-destroying agents was investigated; trypsin, saponin, Liquoid (sodium polyanethol sulphonate), and streptokinase.

Each of the above substances was incorporated in 10 ml. amounts in bile broth in sterile universal containers. One-tenth milliliter volumes of a suitable tenfold dilution of a twelve-hour digest broth culture of a freshly isolated strain of *S. typhi* was added to each bottle along with 3 ml. of fresh whole blood from a healthy donor.

Six 0.02 ml. amounts of the tenfold dilution were spotted onto the surface of dried nutrient agar plates and the initial inoculum calculated after incubation of the plates for eighteen hours.

The universal containers were incubated at 37.0° C. and 0.02 ml. volumes were spotted out as already described at varying intervals of time. In each instance six spots were dropped, and the average colony count developed, after eighteen hours incubation of the plates, was calculated.

TABLE I. COMPARISON OF CULTURE MEDIA FOR GROWTH OF SALMONELLA TYPHI

	TIME OF INCUBATION					
	2 HOURS		6 HOURS		24 HOURS	
	DILUTIONS		DILUTIONS		DILUTIONS	
MEDIA	A	B	A	B	A	B
Bile broth	24	98	P	P	C	C
Selenite F	18	81	140	P	P	P
Tetrathionate broth	10	86	128	P	P	P

P = single colonies too numerous to count.
C = confluent growth.
Dilution A = inoculum of approximately 8.3 organisms per milliliter.
Dilution B = inoculum of approximately 83 organisms per milliliter.
Figures refer to the number of colonies developed per 0.02 ml. volume.

2. *Results:* It will be observed (Table II) that in the control tube containing bile broth alone there was a progressive reduction in the colony count over the twenty-four-hour period. Sampling after 48 hours showed complete sterility. The amount of blood used, 3 ml., gives a dilution of about 1/4.3. This corresponds to a serum dilution of about 1/8.6, which we have previously shown may be adequate to cause complete killing of a small inoculum of *S. typhi.*[4] This is in agreement with the observations of Gerstung,[5] who showed that in a series of 60 combinations of sera with various organisms only 29 yielded growth at 1/12 or greater concentration of serum.

Liquoid produced sufficient destruction of complement to allow satisfactory growth, with all the concentrations employed, as did the higher concentrations of the other three agents. Saponin below a concentration of 0.625 per cent was less satisfactory, as were trypsin in concentrations of less than 0.625 per cent and streptokinase in concentrations of less than 100 units per milliliter.

TABLE II. EFFECT OF COMPLEMENT DESTROYING AGENTS IN BLOOD CULTURE FOR *Salmonella typhi*

SUBSTANCE	CONCENTRATION	TIME OF INCUBATION		
		2 HOURS	6 HOURS	24 HOURS
Trypsin	5%	102	P	C
	2.5%	110	P	C
	1.25%	108	P	C
	0.625%	40	118	C
	0.317%	20	115	C
Saponin	2.5%	124	P	C
	1.25%	116	P	C
	0.625%	68	83	C
	0.317%	18	2	nil
	0.158%	nil	nil	nil
Liquoid	2%	123	P	C
	1%	116	P	C
	0.5%	114	P	C
	0.25%	120	P	C
	0.125%	110	P	C
Streptokinase	400 units/ml.	113	P	C
	200 units/ml.	117	P	C
	100 units/ml.	104	P	C
	50 units/ml.	42	80	C
	25 units/ml.	38	54	C
Bile broth alone		14	4	nil

P = single colonies too numerous to count.
C = confluent growth.
Figures refer to the number of colonies developed per 0.02 ml. volume.

DISCUSSION

We have previously shown that in the isolation of *S. typhi* from whole blood it is advisable to supply a sufficiently large volume of medium in order to dilute out any bactericidal serum factors which may be present.[4] In order for allowance to be made for sampling of a sufficiently large volume of blood, however, there entails the use of large volumes of medium, which may not be practicable. The destruction of complement by some substance which does not affect the viability of any organisms present in the blood will obviously allow smaller volumes of medium to be used. In addition, the agents discussed above have the added advantage of preventing clot formation.

If these agents are to be employed usefully in blood culture methods, then concentrations at least as great as those discussed above will be required. Lower concentrations will not be fully effective where the volume of medium is not sufficient to dilute out any bactericidal serum factors. Particularly will this apply where there is a low grade bacteremia. We have shown that the bactericidal effect of normal serum may be very rapid[4] and complete sterility

may result, with too low a concentration of complement-destroying agent, before there is sufficient destruction of complement to prevent this.

There appears to be little to choose between the various complement-destroying agents discussed above, and they may usefully be employed in all routine blood culture techniques. In the case of streptokinase, occasional sera are encountered with a high anti-streptokinase titer, the result of previous streptococcal infection, and in these cases higher concentrations will be required.

SUMMARY

Investigation of various media has shown that bile salt broth is the most satisfactory medium for isolation of *S. typhi* from the blood stream. The addition of various complement destroying agents to bile broth increases the likelihood of successful isolation where the volume of medium is limited.

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THE DETERMINATION OF PROTEIN-BOUND IODINE^{131} WITH ANION EXCHANGE RESIN COLUMN

THEODORE FIELDS, M.S., DAVID S. KINNORY, PH.D., ERVIN KAPLAN, M.D., Y. T. OESTER, M.D., PH.D., AND EVERETT N. BOWSER, B.S.
HINES, ILL.

THE diagnostic significance of protein-bound iodine^{131} in the examination of thyroid function has previously been recognized and discussed.[1-3] Zieve[4] first determined protein-bound I^{131} in human blood plasma by removing inorganic iodide^{131} from PBI^{131} using Amberlite anion exchange columns. In order to determine the reliability of this method, a direct comparison of this procedure with the trichloroacetic acid precipitation technique was undertaken in our laboratory in one hundred consecutive thyroid function studies. The high degree of correlation between these two methods and the considerable saving in technicians' time afforded by the use of the anion exchange resin recommend this reproducible and simplified procedure as a valuable addition to the techniques of thyroid diagnosis.

METHOD

A tracer dose of 50 μc of carrier-free NaI^{131}* was given orally to the fasting patient. After twenty-four hours, blood for the determination was drawn in a heparin-wetted syringe and two 5 ml. aliquots of plasma were obtained after centrifugation. PBI^{131} was then determined by both the trichloroacetic acid precipitation technique, reported previously,[2] and the anion exchange column method, which is here described.

The anion exchanger used was Amberlite IRA 400 OH,† which was converted to the chloride form by treating it with a sodium chloride solution. This was conveniently accomplished by suspending the resin in a 1 N NaCl solution and pouring the mixture into a large glass column equipped with a sintered glass support and partially opened stopcock. The addition of the sodium chloride solution was continued from a reservoir, in the form of a separatory funnel tightly fitted in the mouth of the column, until the effluent was no longer basic. The excess sodium chloride was removed from the resin by continued washing with distilled water, which flowed from the reservoir, until the effluent failed to form a precipitate upon the addition of a dilute solution of $AgNO_3$. The resin was then spread on absorbent paper, air-dried, and stored in a closed container for future use.

The ion exchange columns were prepared in the following manner: A 21 cm. long glass tubing, 4 mm. internal and 6 mm. external diameter, served as a sleeve for the resin column. A small piece of glass wool was introduced into one end and flamed for a moment to form a porous support for the resin. The other end of the glass tubing was flared out 1 or 2 mm. to receive a plastic round-bottom tube, ½ in. by 3⅞ inches,‡ in the bottom of which was drilled a 5 mm. hole. This plastic tube served as a reservoir for the plasma. The entire column was then assembled by passing the glass tubing through the plastic

From the Radioisotope Unit, Veterans Administration Hospital, Hines, Ill.
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*Abbott Laboratories, Oak Ridge, Tenn.
†Rohm and Haas Company, Philadelphia, Pa.
‡Lusteroid Company, 10 W. Parker Ave., Maplewood, N. J.

tube and applying Duco cement* to the flared end of the glass tubing. This was pressed against the bottom of the plastic tube until a firm seal was obtained, which dried in about four hours. This type of column was used in preference to conventional thistle tubes because its low cost warrants its disposal after use (Fig. 1).

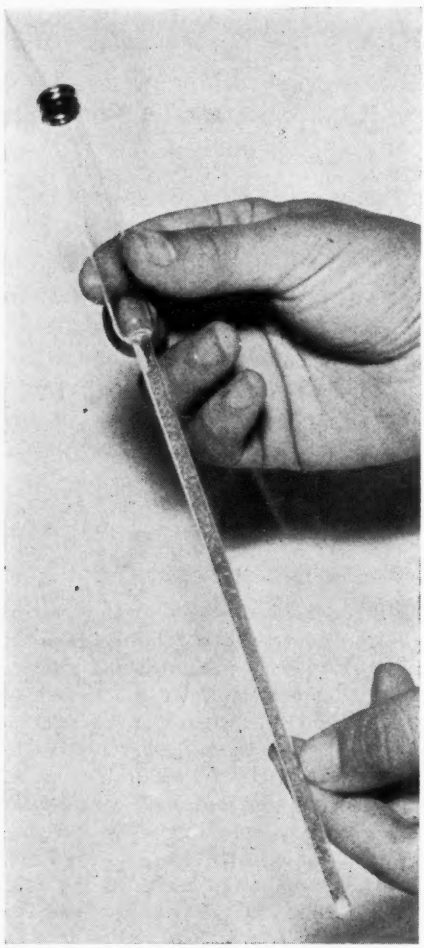

Fig. 1.—The Amberlite anion exchange column.

The glass tubing part of the column was filled with the previously prepared chloride form of Amberlite IRA 400 by introducing the resin into the plastic tube and shaking it into the glass tubing until the entire 21 cm. length of the latter was filled. This required approximately 1.5 Gm. of the resin. Immediately before use, the whole length of the glass tubing was immersed vertically in a beaker of distilled water just long enough to bring about uniform packing and wetting of the resin column. Five milliliters of plasma was then pipetted into the plastic reservoir portion of the column assembly and the effluent, now free of inorganic iodide, was received in a plastic tube containing 1.5 ml. of 40 per cent trichloroacetic acid. After the column had drained dry, it was washed free of any remaining plasma with 2 ml. of distilled water. The contents of the receiving tube were then thoroughly mixed and centrifuged. The supernatant liquid which contained no

*E. I. du Pont de Nemours & Co., Wilmington, Del.

radioactivity was carefully decanted, and the radioactivity of the protein-bound iodine precipitate was measured in a well scintillation counter. The single TCA precipitation reduced the volume of activity in the effluent, thereby increasing the efficiency of counting by a factor of approximately 5 per cent. As a practical time-saving expedient, the effluent may be counted without precipitation. For this study, however, the precipitate of the effluent was obtained so that a direct comparison could be made with TCA method by having similar geometry for counting.

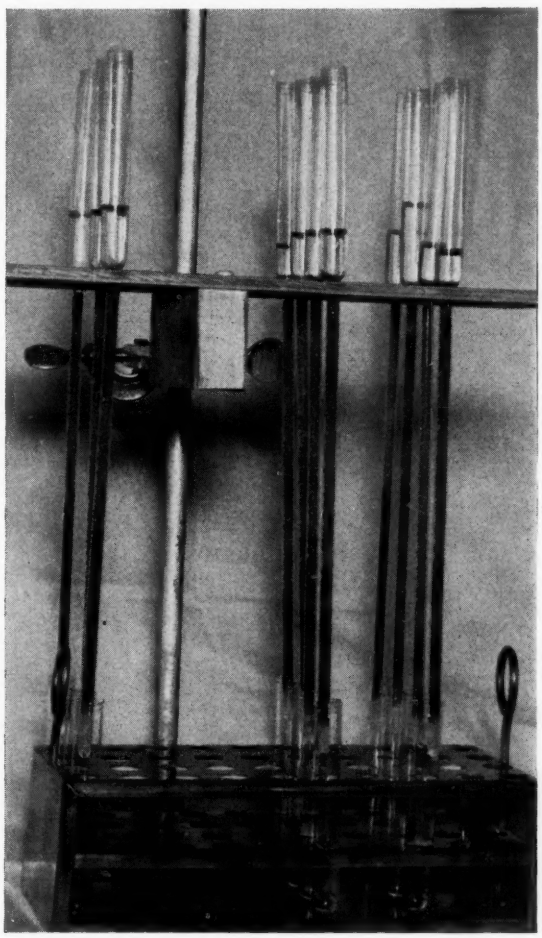

Fig. 2.—Multiple Amberlite anion exchange columns. A demonstration of the simultaneous separation of free I^{131} from PBI^{131} in eleven plasma samples.

As a support for a number of resin columns, a rectangle of perforated plywood is used. The board is 4 inches by 12 inches by ¼ inch thick, with ⅜ inch holes at regular intervals, and held in a clamp on a ring stand. The holes correspond to the spacing of the receiving tubes in the test tube rack placed under it. This arrangement facilitates carrying out a large number of PBI^{131} determinations in an efficient manner (Fig. 2).

RESULTS AND DISCUSSION

Comparison of the TCA and ion exchange methods was made on separate aliquots of plasma from 100 consecutive patients. The average ratio of PBI^{131}

by ion exchange to PBI^{131} by TCA method was 104 per cent. The ranges of values expressed as per cent of administered dose per liter of plasma were comparable to those previously reported.[2, 5] The protein-bound iodine^{131} per liter of plasma of euthyroid patients contained less than 0.128 per cent and that of hyperthyroid patients more than 0.16 per cent of the administered dose I^{131} activity. Included in these 100 cases were 44 patients in whom the probable error of counting was greater than ±14 per cent, by virtue of a low PBI^{131} activity. This probable error is inherent in low activity counting and does not apply specifically to either method. In the remaining 56 patients in whom the statistical error ranged from 1 to 14 per cent, the above ratio of PBI^{131} values was 93.5 per cent, with a counting accuracy of a probable error of ±6.9 per cent.

An interesting suggested application of this technique is the removal of free iodine^{131} from iodinated human serum albumin (RISA*). Since iodide^{131} ions may dissociate from albumin on standing, a source of error ensues in dilution method plasma volume determination. It may be advisable to pass the RISA through a sterile column of the type described immediately before use.

SUMMARY

A direct comparison of the protein-bound iodine^{131} determination by the trichloroacetic acid precipitation technique and the Amberlite anion exchange column procedure was made in 100 consecutive thyroid function studies, and a close agreement between the results of these two methods was established.

The simplified and time-saving procedure for the determination of protein-bound iodine^{131}, employing an Amberlite anion exchange column, is described.

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ADDENDUM

Since this paper was originally submitted, we have found it more convenient to close the tip of the glass column, prior to its filling with resin, by dipping it in molten paraffin wax rather than by inserting a glass wool plug. The top of the column is sealed with about 1 ml. of molten paraffin wax, applied with a medicine dropper directly to the resin, preventing desiccation of the resin. Before use, the wax plugs at both ends of the column are opened by a single perforation with a 19 gauge needle.

*Abbott Laboratories, Oak Ridge, Tenn.